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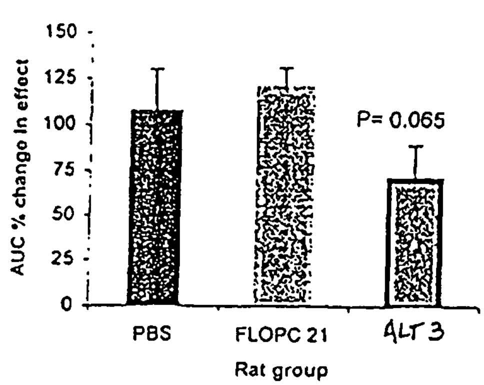
(54) Title: THERAPEUTIC IMMUNE CONDITION COMPOSITIONS AND METHODS



(57) Abstract: Disclosed are compositions for reducing an immune condition, such as inflammation or cancer, in an animal suffering from the immune condition or predisposed to suffer from the immune condition. The compositions comprise a binding agent that specifically binds to an adhesion molecules expressed on the surface of or released by a blood-borne cell or an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Additional disclosed compositions comprise an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount. Also disclosed are methods for administering such compositions to an animal suffering from or predisposed to suffer from the immune condition.

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THERAPEUTIC IMMUNE CONDITION COMPOSITIONS AND METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit from U.S. provisional application serial no. 60/150,652 filed August 25, 1999, the entire contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to compositions for the treatment and/or prevention of immune conditions, particularly inflammation and cancer, and methods for making and using such compositions.

Summary of the Related Art

Two immune conditions that affect the health of humans and domesticated animals are inflammation and cancer. Gallin *et al.* (Inflammation: Basic Principles and Clinical Correlates, Raven Press, New York, 1988) teaches that inflammation is a complex protective physiologic response elicited by various stimuli such as infectious agent, localized tissue injury, or other trauma. The inflammatory response involves numerous mediators and various immune cells. Issekutz *et al.* (*Immunology* **88**: 569-576, 1996) teaches that the migration of white blood cells (*i.e.*, leukocytes) from the blood stream through the endothelial cell wall to sites of injury, infection, or immunological reaction is recognized as a characteristic feature and a critical step of the inflammatory response. The accumulation of leukocytes into inflamed tissues occurs as a consequence of endothelial cell activation and leukocyte migration to the inflammatory foci.

Díaz-González and Sánchez-Madrid (*Immunology Today* **19**(4): 1-3, 1998) teach that the process of leukocyte migration requires an interaction between leukocytes and the endothelial cells lining the blood vessel. Leukocyte-endothelial cell interaction is a multi-step process, which involves a cascade of sequential cellular adhesive events that can be divided into four successive steps as illustrated in Figure 1. The initial contact between

leukocytes and the blood vessel wall is a reversible process that involves rolling of leukocytes on the activated endothelium (step one). This rolling of the leukocytes on endothelial cells is facilitated by the adhesion of a selectin molecule (*e.g.*, L-selectin) on the leukocyte to its ligand (*e.g.*, sialyl Lewis X or Sialyl Lewis A) on the endothelial cell. Rolling of leukocytes is followed by activation of the leukocytes by chemokines and cytokines (step two), and firm adhesion of activated leukocytes to the endothelium (step three). Crockett-Torabi and Fantone (*Immunol. Res.* **14**: 237-251, 1995) teach that in the last step, leukocytes extravasate into the surrounding tissues by squeezing themselves between endothelial cell junctions and moving toward the inflammatory foci.

The majority of inflammatory disorders are frequently chronic in duration, and of particular importance are the autoimmune diseases. Autoimmune disease is a group of chronic diseases, in which the immune system not only fail to perform its regular function in protecting the host from invading agents, but also reacts particularly against some of the host autoantigens. This autoimmune response is initiated by cellular, humoral, or both mechanisms, and leads to disease with local inflammation and tissue damage. Multiple sclerosis (MS), systemic lupus erythematosus (SLE), Crohns disease, and rheumatoid arthritis (RA) are just few examples of autoimmune diseases.

Pitzalis *et al.* (*Annals of Rheumatic Diseases* **53**: 287-288, 1994) discuss the role of adhesion molecules in the pathogenesis of RA and other autoimmune diseases. Leukocyte infiltration into tissues is believed to be a major component of the pathologic process leading to joint injury in chronic arthritis, including rheumatoid arthritis. Therefore, adhesion molecules represent another attractive immunotherapeutic target for intervention in RA and other autoimmune diseases.

Selectins and their carbohydrate ligands have been considered very important targets in inflammatory conditions because of their important role in the initial contact between leukocytes and the vascular endothelium at sites of inflammation. McMurray, R.W. (*Seminars in Arthritis & Rheumatism* **25**: 215-234, 1996) teaches that selectins are carbohydrate binding adhesion molecules that share a common structural component consisting of a $Ca^{(2+)}$ dependent N-terminal lectin binding domain. McEver, R.P. (*Curr Opin Immunol* **6**: 75-84, 1994) teaches that selectins recognize and bind to specific carbohydrate structures on ligands that are composed of sialic acid, fucose, galactose, mannose, and/or an anionic sulfate or phosphate ester moieties such as sialyl Lewis X (SLe(x)), sialyl Lewis A (SLe(a)), and related structures.

Munro *et al.* (*American Journal of Pathology* **141**(6): 1397-1405, 1992) teach that the carbohydrate ligand, SLe(x)) [NeuAca2-3Galb1-4(Fuca1-3) GlcNAc-] is expressed on the cell surface of monocytes, a polymorphonuclear leukocyte (PMNL), and on approximately 10% or peripheral blood T lymphocytes. SLe(x) is recognized by all selectin adhesion molecules. The SLe(x) isomeric epitope, SLe(a) [NeuAca2-3Galb1-3(Fuca1-4) GlcNAc-] (Zhang *et al.*, *Tumor Biology* **18**: 175-187, 1997), is recognized and bound to by E and P-selectins, and possibly L-selectin (Paavonen and Renkonen, *American Journal of Pathology* **141**(6): 1259-1261, 1992). Takada *et al.* (*BiochemBiophys Res Commun* **179**: 713-19, 1991) teach that SLe(a) is absent on most peripheral blood cells.

Blocking selectins or their ligands with antibodies or oligosaccharides has been attempted in various animal models of inflammation. For example, Weiser *et al.* (*Shock* **5**(6): 402-407, 1996) describe that in rats that had undergone hour of hindlimb tourniquet ischemia, the administration of anti-P-selectin monoclonal antibody (mAb), PB1.3, allegedly reduced hindlimb injury, but not leukosequestration. Yamada *et al.* (*European Journal of Pharmacology* **346**: 222-224, 1998) describe that the same anti-P-selectin mAb PB1.3 allegedly reduced infarct size by approximately 38% (2 mg/kg) and 28% (5 mg/kg) in rabbits undergoing 30 minutes of ischemia followed by 5 hour reperfusion. Tojo *et al.* (*Glycobiology* **6**(4): 463-9, 1996) allege that the anti-rat-P-selectin mAb, ARP2-4, attenuated infarct size in rat myocardial ischemia and reperfusion injury model. Ohnishi *et al.* (*Immunopharmacology* **34**: 161-170, 1996) describe that the anti-rat-P-selectin mAb, ARP2-4, allegedly reduced the footpad swelling and inhibited the associated polymorphonuclear leukocyte (PMNL) accumulation when administered to rats who had undergone dermal injury induced by Arthus reaction.

Altavilla *et al.* (*European Journal of Pharmacology* **270**: 45-51,1994) allege that the use of the anti-E-selectin mAb, BBIG-E5, resulted in the reduction in infarct size as well as PMNL accumulation in rodent model.

A study on endotoxin-induced uveitis in mice (Whitcup *et al.*, *Clinical Immunology and Immunopathology* **83**(1): 45-52, 1997) reported that neutrophil migration into the eye was allegedly effectively inhibited with a combination of anti- E-selectin and anti-P-selectin monoclonal antibodies administered intraperitoneally, either before or after endotoxin injection. In this study, anti-E-selectin antibody alone allegedly had little effect on endotoxin-induced uveitis, while anti-P-selectin mAb decreased ocular inflammation by 37% when administered at the time of or 6 hours after endotoxin injection.

Ma *et al.* (*Circulation* **88**: 649-658, 1993) allege that a reduction in infarct size as well as PMNL accumulation was observed with the use anti-L-selectin mAb, DREG-200, in a feline model. Pizcueta and Luscinskas (*American Journal of Pathology* **145**(2): 461-467, 1994) disclose an *in vivo* murine model of experimentally induced chronic inflammation of the peritoneum in which anti-L-selectin mAb (MEL-14) was infused intravenously (i.v.) to investigate the role of L-selectin in the recruitment of mononuclear leukocytes to chronic sites of inflammation (48 hours). Results from this study showed an allegedly reduced accumulation of macrophages and lymphocytes by 60% and 90%, respectively, at 48 hours. Similarly, MEL-14 mAb allegedly dramatically inhibited granulocyte influx by 80% at 6 hours and by 50% at 24 and 48 hours.

Most of the studies on selectin-carbohydrate ligands have targeted Sialyl Lewis X (SLe(x))) oligosaccharide molecule. Tojo *et al.* (*Glycobiology* **6**(4): 463-9, 1996) describe the effect of SLe(x)-oligosaccharide on rat myocardial ischemia and reperfusion injury. In this study, the administration of SLe(x)-oligosaccharide allegedly caused significant reduction in infarct size without affecting hemodynamic parameters or circulating leukocyte numbers.

Han *et al.* (*Journal of Immunology* **155**(8): 4011-5, 1995) describe the effect of a soluble SLe(x) on ear edema and necrosis in rabbits where the ear vascular supply was occluded for 6 hours, and allowed to reperfuse later. In this study, tissue edema and necrosis were allegedly significantly reduced in animals treated with SLe(x) (25 mg / kg bolus i.v. followed by 50 mg/kg infusion over 10 hour) immediately upon reperfusion or after a 1-hour delay, but not in animals for whom the treatment was delayed 4 or 12 hours.

Schmid *et al.* (*Journal of Heart & Lung Transplantation* **16**(10): 1054-1061, 1997) evaluated the inhibitory effect of CY-1503, an analogue of SLe(x), on PMNL migration and reperfusion injury in canine left lung allografts (35 mg/kg i.v. bolus) where the recipient contralateral right pulmonary artery and bronchus were ligated. In this study, allograft gas exchange and hemodynamics results allegedly demonstrated that the group treated with CY-1503 had a reduction in PMNL adhesion, migration, and subsequent reperfusion injury in preserved canine lung allografts. Birnbaum *et al.* (*Journal of Molecular & Cellular Cardiology* **29**(8): 2013-25, 1997) discloses that the treatment with CY-1503 (30 mg/kg i.v. bolus) allegedly did not limit infarct size or prevent the "no-reflow" phenomenon in rabbits who were subjected to 30 minutes of coronary artery occlusion and 4 hour of reperfusion. Yet additional studies demonstrated that SLe(x) oligosaccharide allegedly reduced infarct

size by 83% and inhibited PMNL adhesion to endothelial cells in the feline model (Buerke *et al.*, *Journal of Clinical Investigation* **93**: 1140-1148, 1994); and in the canine model it allegedly reduced infarct size by 67% and ameliorated PMNL accumulation in myocardium by 63% (Lefer *et al.*, *Circulation* **90**: 2390-2401, 1994).

In addition, the effect of anti- SLe(x) mAb and the expression of SLe(x) were examined in rat hearts subjected to 30 minutes of ischemia followed by reperfusion (Yoshinori *et al.*, *Journal of Pathology* **180**: 305-310, 1996). Reperfusion of ischaemic myocardial tissue in this study allegedly resulted in enhanced expression of SLe(x) on the luminal surface of vascular endothelial cells, as well as myocytes. Furthermore, the *in vivo* administration of anti- SLe(x) mAb significantly reduced the extent of the myocardial infarction.

The mortality from cancer often arises not from the primary tumor site, but from the secondary tumor sites caused by migration of metastatic cancer cells. Many cancer cells, like many blood cells, express sialyl Lewis X and/or sialyl Lewis A (Ravindranath et al., Cancer **79(9)**:1686-1697, 1997; Tomlinson *et al.*, *Int. J. Oncol.* **16(2)**:347-353, 2000; Skorstengaard *et al.*, *J. Urol.* **161(4)**:1316-1323, 1999; Groves *et al.*, *Am. J. Pathol.* **143(4)**:1220-1225, 1993; Farmer *et al.*, *Head Neck* **20(8)**:726-731, 1998)

Fukuda *et al.*, *Cancer Res.* **60(2)**:450-456, 2000) allege that a peptide mimic of E-selectin ligand inhibits the lung colonization of melanoma cells expressing sialyl Lewis X.

Although numerous studies have attempted to use antibodies against adhesion molecules to reduce immune conditions in animal models, because such high dosages of antibody are required, these approaches may result in antibody-dependent cellular cytotoxicity (ADCC) or antibody-mediated toxicity in human patients. Thus, there remains a need to develop safer approaches to reducing immune conditions that allow for the administration of lower dosages of binding agents, such as antibodies, to adhesion molecules.

SUMMARY OF THE INVENTION

The invention provides compositions for reducing an immune condition. In addition, the invention provides methods of administration of an agent, preferably a binding agent (such as an antibody) to an adhesion molecule, particularly an adhesion molecule involved in the extravasion of white blood cells or cancer cells from the blood into the surrounding tissue, to an animal suffering from the immune condition (or predisposed to develop the immune condition). The relatively low dosage of the agent according to the compositions and methods of the invention allow for a reduction in the risk of a treated animal developing antibody-dependent cellular cytotoxicity (ADCC) or antibody-mediated toxicity.

Accordingly, in a first aspect, the invention provides a method for reducing an immune condition in an animal suffering from the immune condition, comprising administering to the animal a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the animal is a domesticated. More preferably, the animal is a mammal, such as a human.

In some embodiments of the first aspect of the invention, the adhesion molecule is a molecule comprising a carbohydrate epitope. In certain embodiments, the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A. In some embodiments, the adhesion molecule is a selectin. In preferred embodiments, the selectin is P-selectin or E-selectin.

In certain embodiments of the first aspect of the invention, the immune condition is inflammation. In certain embodiments, the blood-borne cell is a white blood cell. In certain embodiments, the white blood cell is selected from the group consisting of a lymphocyte, a neutrophil, a polymorphonuclear leukocyte, and a monocyte.

In certain embodiments of the first aspect of the invention, the immune condition is cancer. In certain embodiments, the blood-borne cell is a cancer cell. In certain embodiments, the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

In various embodiments of the first aspect, the binding agent is an antibody, such as a monoclonal antibody. Preferably, the binding agent is a murine monoclonal antibody.

In certain embodiments of the first aspect of the invention, the immune response results in the generation of an antibody that specifically binds to the binding agent. In certain embodiments, the immune response results in the generation of an antibody that specifically binds to the adhesion molecule. In some embodiments, the immune response comprises a humoral and a cellular immune response.

In various embodiments of the first aspect, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

In a second aspect, the invention provides a composition for reducing an immune condition in an animal suffering from the immune condition comprising a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the animal is a domesticated. More preferably, the animal is a mammal, such as a human.

In some embodiments of the second aspect of the invention, the adhesion molecule is a molecule comprising a carbohydrate epitope. In certain embodiments, the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A. In some embodiments, the adhesion molecule is a selectin. In preferred embodiments, the selectin is P-selectin or E-selectin.

In certain embodiments of the second aspect of the invention, the immune condition is inflammation. In certain embodiments, the blood-borne cell is a white blood cell. In certain embodiments, the white blood cell is selected from the group consisting of a lymphocyte, a neutrophil, a polymorphonuclear leukocyte, and a monocyte.

In certain embodiments of the second aspect of the invention, the immune condition is cancer. In certain embodiments, the blood-borne cell is a cancer cell. In certain embodiments, the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

In various embodiments of the second aspect, the binding agent is an antibody, such as a monoclonal antibody. Preferably, the binding agent is a murine monoclonal antibody.

In certain embodiments of the second aspect of the invention, the immune response results in the generation of an antibody that specifically binds to the binding agent. In certain embodiments, the immune response results in the generation of an antibody that specifically binds to the adhesion molecule. In some embodiments, the immune response comprises a humoral and a cellular immune response.

In various embodiments of the second aspect, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

In a certain preferred embodiment of the second aspect of the invention, the composition also comprises a pharmaceutically-acceptable carrier.

In a third aspect, the invention provides a therapeutic composition comprising an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount. Preferably, the agent is a binding agent.

In a fourth aspect, the invention provides a method for reducing an immune condition in an animal suffering from cancer or inflammation or predisposed to suffer from cancer and inflammation, comprising administering to the animal a therapeutically effective amount of an agent that is capable of reducing inflammation and reducing cancer in an animal Preferably, the agent is a binding agent.

According to the invention, agents of the invention, such as binding agents found to specifically bind to an adhesion molecule, may be administered in a therapeutically effective amount to an animal suffering from inflammation, cancer, or predisposed to suffer from inflammation or cancer. For example, a therapeutically effective amount of an antibody that specifically binds to Sialyl Lewis X may be administered with a pharmaceutically-acceptable carrier (*e.g.*, physiological sterile saline solution) via any route of administration to a patient

suffering from inflammation in an attempt to alleviate any resulting disease symptom (*e.g.*, necrosis). For example, the agent of the invention may be delivered subcutaneously, intravenously, intraperitoneally, intra-arterially, intradermally, or intra-muscularly. Pharmaceutically-acceptable carriers and their formulations are well-known and generally described in, for example, Remington's Pharmaceutical Sciences (18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation illustrating the different steps in the adhesion cascade between leukocytes and endothelial cells (Díaz-González and Sánchez-Madrid, *Immunology Today* **19**(4): 1-3, 1998).

Figure 2 is a schematic representation of the idiotypic cascade, a non–limiting theory of the present invention, showing reactivity with ligand and/or receptor by mirror image. In this schematic, Ab1 (the injected antibody) which specifically binds to and blocks the receptor antigen on the surface of a cell (*e.g.*, a tumor cell) is depicted as having a diamond shape. The injected animal then generates an immune response to Ab1 resulting in the production of Ab2, depicted in this schematic as having a "Y" shape. Ab2 in this schematic specifically binds to the natural ligand (depicted as a solid triangle) of the receptor. The presence of Ab2 results in the production by the immune system of the animal a third antibody, Ab3, which is depicted as having a pentagonal shape that mimicks the natural ligand of the receptor.

Figure 3 is a schematic representation illustrating the use of the enzyme linked immunosorbent assay (ELISA) for the detection of Ab2 antibodies. Note that where the Ab1 antibody is a murine antibody which is injected into a rat, this ELISA will also detect rat anti-mouse antibodies (RAMA).

Figure 4 is a schematic representation illustrating the use of ELISA for the detection of Ab3. Note that the CA19-9 antigen bears the Sialyl Lewis A antigen.

Figure 5 is a schematic representation showing SDS-polyacrylamide Gel Electrophoresis of purified monoclonal antibodies (10% acrylamide Tris-HCl under reducing conditions). Molecular weight (MW) is indicated at the left for the standard loaded into Lane 1. The antibodies are as follows: F5 (IgM) (Lane 2; purified by Mannan binding protein affinity chromatography); 6C2C5C12 (IgM) (Lane 3; purified by the euglobulin precipitation method); ALT-4 (also called HB1; IgM) (Lane 4; purified by Mannan binding protein affinity chromatography); Alt-4 (also called HB1; IgM) (Lane 5; old sample of purified antibody); Flopc-21 (IgG3) (Lane 6; purified by protein A affinity chromatography) ALT-3 (IgG3) (Lane 7; purified by the euglobulin precipitation method); and ALT-3 (IgG3) (Lane 8; old sample of purified antibody).

Figure 6 is a schematic representation showing the binding of various representative, non-limiting murine monoclonal antibodies (7C2C5C12, F5, ALT-3, and Flopc-21 (at the

indicated concentrations) to immobilized tumor antigen CA19-9, a non-limiting representative antigen of the invention.

Figure 7 is a schematic representation showing the binding of various representative, non-limiting murine monoclonal antibodies (7C2C5C12, F5, ALT-3, and Flopc-21 (at the indicated concentrations) to immobilized polyacrylamide-sialyl Lewis A oligosaccharide, a non-limiting representative antigen of the invention.

Figure 8 is a schematic representation showing the inhibitory effect of sialyl Lewis A antigen, a non-limiting representative antigen of the invention, on the binding of Iodine-125 radiolabeled HB1 (also called ALT-4) antibody, a non-limiting representative therapeutic antibody of the invention, to immobilized CA19-9 antigen, a non-limiting antigen of the invention. Radio-immunoassay plates were coated with CA19-9 at 65 U/mL and incubated with radiolabeled HB1 at 0.33 μg/mL (a non-saturating amount) and increasing concentrations of Sialyl Lewis A antigen (0-100 μg/ml).

Figure 9 is a schematic representation showing Scatchard analysis of the binding of Iodine-125 radiolabeled HB1 mAb (also called ALT-4), a non-limiting representative therapeutic monoclonal antibody of the invention, to CA19-9 antigen, a non-limiting representative antigen of the invention. Radioimmunoassay strips were coated with CA19-9 antigen at 65 U/mL, and radiolabeled antibody was added to strips at different dilutions ranging from 0.0005 μg/mL to 10.5 μg/mL. The affinity of HB1 antibody to CA19-9 was determined by measuring the radioactivity of free and bound fractions in individual wells by Gamma counter.

Figures 10A, 10B and 10C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the caliper method. Eight measurements were performed, and the results are represented as AUC percent change in effect. Figures 10A, 10B and 10C represent the same data, illustrated as a bar graph (Fig. 10A), a column scatter (Fig. 10B), and a whicker box (Fig. 10C).

Figures 11A, 11B, and 11C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 11A), a column scatter (Fig. 11B), and a whicker box (Fig. 11C).

Figures 12A, 12B, and 12C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the water displacement method. Eight measurements were performed, and the results are represented as AUC percent change in effect. Figures 12A, 12B and 12C represent the same data, illustrated as a bar graph (Fig. 12A), a column scatter (Fig. 12B), and a whicker box (Fig. 12C).

Figures 13A, 13B, and 13C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the water displacement method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 13A), a column scatter (Fig. 13B), and a whicker box (Fig. 13C).

Figure 14 is a schematic representation of a column scatter graph showing the RAMA response with the inflammatory response in different rat groups for Example I. Figs. 10B and 12B are reiterated below Fig. 14 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 10B) or the water displacement method (Fig. 12B) with the level of immune response (Fig. 14)

Figures 15A, 15B and 15C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by caliper method. Eight measurements were performed, and the results are represented as AUC percent change. Figures 15A, 15B and 15C represent the same data, illustrated as a bar graph (Fig. 15A), a column scatter (Fig. 15B), and a whicker box (Fig. 15C).

Figures 16A, 16B, and 16C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 16A), a column scatter (Fig. 16B), and a whicker box (Fig. 16C).

Figures 17A, 17B, and 17C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by water displacement method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 17A), a column scatter (Fig. 17B), and a whicker box (Fig. 17C).

Figures 18A, 18B, and 18C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by water displacement method.

Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 18A), a column scatter (Fig. 18B), and a whicker box (Fig. 18C).

Figure 19 is a schematic representation of a column scatter graph showing the RAMA response with the inflammatory response in different rat groups for Example II. Figs. 15B and 17B are reiterated below Fig. 19 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 15B) or the water displacement method (Fig. 17B) with the level of immune response (Fig. 19)

Figures 20A-20H are schematic representations of line graphs showing the correlation between the RAMA response and the inflammatory response at different time measurements in the group of rats treated with HB1 antibody, a non-limiting representative therapeutic antibody of the invention, in Example II. The inflammatory response was measured at 1.5 hours (Fig. 20A), 2.5 hours (Fig. 20B), 3.5 hours (Fig. 20C), 4.5 hours (Fig. 20D), 5.5 hours (Fig. 20E), 6.5 hours (Fig. 20F), or 7.5 hours (Fig. 20G) after injection of carrageenan. In Fig. 20H, the AUC percent change in effect was plotted against the RAMA (Ab2) response.

Figures 21A, 21B and 21C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the caliper method. Seven measurements were performed, and the results are represented as AUC percent change in effect. Figures 21A, 21B, and 21C represent the same data, illustrated as a bar graph (Fig. 21A), a column scatter (Fig. 21B), and a whicker box (Fig. 21C).

Figures 22A, 22B, and 22C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the caliper method. Seven measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 22A), a column scatter (Fig. 22B), and a whicker box (Fig. 22C).

Figures 23A, 23B, and 23C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the water displacement method. Seven measurements were performed, and the results are represented as AUC percent change in effect. Figures 23A, 23B, and 23C represent the same data, illustrated as a bar graph (Fig. 23A), a column scatter (Fig. 23B), and a whicker box (Fig. 23C).

Figures 24A, 24B, and 24C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the water displacement

method. Seven measurements were performed, and the results are represented as percent change in maximum effect. Figures 24A, 24B and 24C represent the same data, illustrated as a bar graph (Fig. 24A), a column scatter (Fig. 24B), and a whicker box (Fig. 24C).

Figure 25 is a schematic representation of a column scatter graph showing the RAMA response with the inflammatory response in different rat groups for Example III. Figures 21B and 23B are reiterated below Figure 20 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 21B) or the water displacement method (Fig. 23B) with the level of immune response (Fig. 25)

Figures 26A-26G are schematic representations showing the correlation between the RAMA response and the inflammatory response at different time measurements in the group of rats treated with HB1 antibody, a non-limiting representative therapeutic antibody of the invention, in Example III. The inflammatory response was measured at 1.5 hours (Fig. 26A), 2.5 hours (Fig. 26B), 3.5 hours (Fig. 26C), 4.5 hours (Fig. 26D), 5.5 hours (Fig. 26E), 6.5 hours (Fig. 26F), or 7.5 hours (Fig. 26G) after injection of carrageenan.

Figures 27A-27G are schematic representations showing the correlation between the RAMA response and the inflammatory response at different time measurements in the group of rats treated with F5 antibody, a non-limiting representative antibody of the invention, in Example III. The inflammatory response was measured at 1.5 hours (Fig. 27A), 2.5 hours (Fig. 27B), 3.5 hours (Fig. 27C), 4.5 hours (Fig. 27D), 5.5 hours (Fig. 27E), 6.5 hours (Fig. 27F), or 7.5 hours (Fig. 27G) after injection of carrageenan.

Figures 28A, 28B and 28C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by caliper method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 28A), a column scatter (Fig. 28B), and a whicker box (Fig. 28C).

Figures 29A, 29B, and 29C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 29A), a column scatter (Fig. 29B), and a whicker box (Fig. 29C).

Figures 30A, 30B, and 30C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by water displacement method.

Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 30A), a column scatter (Fig. 30B), and a whicker box (Fig. 30C).

Figures 31A, 31B, and 31C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by water displacement method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 31A), a column scatter (Fig. 31B), and a whicker box (Fig. 31C).

Figure 32 is a schematic representation of a column scatter graph showing the RAMA (Ab2) response with the inflammatory response in different rat groups for Example IV. Figs. 28B and 30B are reiterated below Fig. 32 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 28B) or the water displacement method (Fig. 30B) with the level of immune response (Fig. 32)

Figures 33A, 33B and 33C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by caliper method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 33A), a column scatter (Fig. 33B), and a whicker box (Fig. 33C).

Figures 34A, 34B, and 34C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 34A), a column scatter (Fig. 34B), and a whicker box (Fig. 34C).

Figures 35A, 35B, and 35C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by water displacement method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 35A), a column scatter (Fig. 35B), and a whicker box (Fig. 35C).

Figures 36A, 36B, and 36C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by water displacement method. Eight measurements were performed, and the results are represented as percent change in

maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 36A), a column scatter (Fig. 36B), and a whicker box (Fig. 36C).

Figure 37 is a schematic representation of a column scatter graph showing the RAMA (Ab2) response with the inflammatory response in different rat groups for Example V. Figs. 33B and 35B are reiterated below Figure 37 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 33B) or the water displacement method (Fig. 35B) with the level of immune response (Fig. 37)

Figures 38A-38H are schematic representations of histograms showing the binding of therapeutic monoclonal antibodies to human neutrophils as determined by single step indirect immunofluorescence flow cytometry (FACS). In Figs. 38A and 38B, colo 205 cells, which are known to express the Sialyl Lewis A epitope, were used as a positive control. Figs. 38C-38H are neutrophils stained with control (no primary antibody) (Fig. 38C), ALT-4 IgM antibody (Fig. 38D), ALT-3 IgG3 antibody (Fig. 38E), 7C2C5C12 IgM antibody (Fig. 38F), F5 IgM antibody (Fig. 38G), and Flopc-21 IgG3 antibody (Fig. 38H).

Figures 39A and 39B are schematic representations showing the instrumental setup and the schematic diagram of the *in vitro* inhibition of leukocyte rolling and adhesion in microchips channels coated with selectins.

Figure 40 is a schematic representation of a bar graph showing the inhibition of human polymorphonuclear leukocyte (PMNL) rolling in microchip channels coated with recombinant E-selectin, a non-limiting representative adhesion molecule of the invention, by the various indicated agents. The graph bars show the percentage of human PMNL rolling.

Figure 41 is a schematic representation showing the inhibition of human PMNL rolling in microchip channels coated with recombinant P-selectin, a non-limiting representative adhesion molecule of the invention, by the various indicated agents. The graph bars show the percentage of human PMNL rolling.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have developed methods and therapeutic compositions for reducing inflammation. The methods and therapeutic compositions of the invention are useful as analytical tools and as therapeutic tools. The invention also provides methods and therapeutic compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects. Standard reference works setting forth the general principles of the technology described herein include Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1994; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, New York, 1989; Kaufman *et al.* (Eds.), Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton, 1995; and McPherson, Ed., Directed Mutagenesis: A Practical Approach, IRL Press, Oxford, 1991. Standard reference works setting for the general principles of immunology and inflammation include Gallin *et al.*, Inflammation: Basic Principles and Clinical Correlates, Raven Press, New York, 1988; Kuby, J., Immunology, 3rd ed., W.H. Freeman, New York, 1997; Coligan *et al.* (Eds.), Current Protocols in in Immunology, John Wiley & Sons, New York, 1991; and Hurley, J.V., Acute Inflammation, 2nd ed., Churchill Livingstone, New York, 1983.

The published patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, published foreign patent applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. Any inconsistency between these publications and the present disclosure shall be resolved in favor of the present disclosure.

In a first aspect, the invention provides a method for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition, comprising administering to the animal a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the binding agent is administered with a pharmaceutically-acceptable carrier.

In a second aspect, the invention provides a therapeutic composition for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition, comprising a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the composition further comprises a pharmaceutically-acceptable carrier.

In a third aspect, the invention provides a therapeutic composition comprising an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount. Preferably, the composition further comprises a pharmaceutically-acceptable carrier.

In a fourth aspect, the invention provides a method for reducing an immune condition in an animal suffering from cancer or inflammation or predisposed to suffer from cancer and inflammation, comprising administering to the animal a therapeutically effective amount of an agent that is capable of reducing inflammation and reducing cancer in an animal.

As used herein, by "agent" is meant a molecule of any size which may be naturally occurring or synthetic. Thus, an agent includes, without limitation, a chemical, a protein, a carbohydrate, a lipid, a nucleic acid, an acid, a base, a synthetic polymer, and a resin.

As used herein, by "immune condition" is meant cancer and/or inflammation. By "predisposed to suffer from an immune condition " is meant an animal that has a genetically or environmentally caused predisposition to suffer from an immune condition, but is not yet suffering from the immune condition. For example, the animal may have a certain type of MHC haplotype rendering it more susceptible to develop an immune condition, such as rheumatoid arthritis, but has not yet developed the condition. Another example is an animal which has been exposed to intense sunlight, but has not yet developed a sunburn. Another example is an animal which has been exposed to a carcinogen, but has not yet developed cancer.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the immune condition is cancer. In accordance with the invention, by "cancer" is meant the excessive growth of an abnormal cell in an animal resulting in the migration of metastatic

cells via the blood stream from the original anatomical site of the original abnormal cell to other parts in the body. In accordance with the invention, the detection of a secondary site of cancer caused by the migration of a metastatic cell is not required for an animal to be diagnosed as suffering from cancer. Rather, an animal (*e.g.*, a human) may have a cell growth biopsied. Upon a physician's inspection of the biopsied tissue and an assessment that the biopsied cells are likely malignant (*i.e.*, capable of metastasis), the animal may be treated in accordance with the invention.

One non-limiting cancer of the invention is melanoma. Other non-limiting cancers of the invention include breast cancer, colorectal cancer, bladder cancer, leukemia, lymphoma, cervix cancer, prostate cancer, testicular cancer, liver cancer, lung cancer, ovarian cancer, and pancreatic cancer.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the immune condition is inflammation. In accordance with the invention, by "inflammation" is meant a local response to cellular injury that is marked by any or all of capillary dilatation, leukocyte infiltration, redness, heat, and pain and that serves as a mechanism initiating the elimination of the inflammation-inducing antigen and of damaged tissue. The term includes acute inflammation, chronic inflammation, and excessive inflammation, such as that leading to inflammatory disease.

Acute inflammation exhibits a rapid onset and is of short duration. The characteristic signs of an acute localized inflammatory response generally include swelling, redness, heat, pain and loss of function (Kuby, J., Immunology, 3rd ed., W.H. Freeman, New York, pages 361-377, 1997). Infiltration of leukocytes into the tissue peaks within the first 6 hours of an acute inflammatory response, and most of them disappear from the inflamed area within 24-48 hours (Kuby, J., *supra*).

Chronic inflammation develops during persistence of an antigen due to infection or various pathologic conditions. The characteristic hallmark of chronic inflammation is the formation of granuloma. Many different types of cells may be found in the extravascular tissues in areas of chronic inflammation, including any or all of neutrophils, eosinophils, macrophages, epithelioid cells, plasma cells, lymphocytes, and fibroblasts (Hurley, J.V., Acute Inflammation, 2nd ed., Churchill Livingstone, New York, pages 135-144, 1983).

Excessive inflammation, either secondary to abnormal recognition of host tissue as "foreign" or deviation from an otherwise normal inflammatory response, leads to

inflammatory disease (Gallin *et al.*, Inflammation: Basic Principles and Clinical Correlates, Raven Press, New York, 1988). Most forms of acute and chronic inflammation are amplified as well as propagated as a result of the recruitment of humoral and cellular response (Gallin *et al.*, *supra*).

As used herein, by "reducing an immune condition" is meant the ability of an agent (e.g., a binding agent) of the invention to alleviate the symptoms of and/or prevent the onset of an immune condition in an animal suffering from the immune condition (or predisposed to suffer from the immune condition (*e.g.*, an animal exposed to the sun which has not yet developed a sunburn; an animal who has been exposed to a carcinogen which has not yet developed cancer; or an animal genetically predisposed to develop an immune condition which has not yet developed the immune condition)) when administered to that animal. Preferably, the alleviation of symptoms and/or prevention of onset is reduced in a treated animal as compared to the immune condition in an animal to which an agent of the invention has not been administered. Preferably, the reduction in the immune condition in an animal to which has been administered a binding agent of the invention is at least 10% lower as compared to an untreated animal; more preferably, the reduction is at least 25% lower; still more preferably, the reduction is at least 50% lower; even more preferably, the reduction is at least 75% lower; and most preferably, the reduction in the immune condition in an animal to which has been administered a binding agent of the invention is at least 100% lower as compared to an untreated animal (*i.e.*, an animal to which a binding agent of the invention has not been administered). Methods for determining the amount of an immune condition in an animal, where the condition is inflammation, include, without limitation, visual detection of redness and measurement of swelling by the caliper method or by the water displacement method described below. Methods for determining the amount of an immune condition in an animal, where the condition is cancer, include, without limitation, detection of the presence of a metastatic cancer cell and detection of cancer cell-induced angiogenesis.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the animal suffering from an immune condition is preferably a domesticated animal including, without limitation, domesticated fowl (*e.g.*, ducks, geese, chickens, turkeys, Cornish hens, and ostriches), domesticated livestock (*e.g.*, cattle, llamas, elephants, camels, pigs, and sheep), domesticated pets (*e.g.*, horses, cats, dogs, ferrets, hamsters, and guinea pigs); and laboratory animals (*e.g.*, primates (*e.g.*, baboons, Rhesus monkeys) and rodents (*e.g.*, mice, rats)).

In a preferred embodiment of the first, second, third, and fourth aspects of the invention, the animal suffering from the immune condition is a mammal. In a preferred embodiment of the first and second aspects of the invention, the animal suffering from the immune condition is a human.

As used herein, by the term, "induces an immune response" is meant that a cellular and/or humoral immune response is induced or increased in an animal to which has been administered a binding agent of the invention, such that the immune response is directed toward the administered binding agent. Thus, in preferred embodiments of the first, second, third, and fourth aspects of the invention, the immune response comprises both a humoral and a cellular immune response.

The presence of such an immune response can be detected by any of the well known immune response detection methods including, without limitation, detection of the presence of a T cell (*e.g.*, a cytotoxic T cell or helper T cell) whose T cell receptor specifically binds to the binding agent and/or to the adhesion molecule (in the context of the major histocompatibility complex), detection of the presence of a B cell whose B cell receptor (*i.e.*, surface-expressed IgM) specifically binds to the binding agent and/or to the adhesion molecule, and detection of the presence of an antibody (of any serotype) which specifically binds to the binding agent and/or to the adhesion molecule.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the immune response results in the generation of an antibody that specifically binds to the binding agent. Such an antibody that specifically binds to the binding agent is generated as a result of an immune response in the treated animal to the administered binding agent. One non-limiting theory explaining such an immune response is the idiotypic cascade first described by Jerne (Jerne, N.K., *Annals of Immunology* **125C**: 373-389, 1974). This theory, as illustrated for an idiiotypic cascade of antibodies, where the first antibody specifically binds to a tumor associated antigen, is illustrated on Figure 2 (Kingsbury *et al.*, *Leukemia* **12:** 982-991, 1998).

Adapting this theory to the present invention, an administered binding agent (*e.g.*, an antibody) that specifically binds to an adhesion molecule is designated Ab1. Ab1 reacts with epitopic determinants on the adhesion molecule. The animal to which has been administered a binding agent of the invention generates an immune response to that binding agent. Where the immune response comprises antibodies that bind to the binding agent, these antibodies are

designated Ab2. Some Ab2 antibodies (namely the Ab2β, recognize the adhesion molecule-binding site of Ab1 and resemble the original adhesion molecule epitope recognized by Ab1. Other Ab2 antibodies recognize sites present elsewhere on the variable region, and may (Ab2γ) or may not (Ab2α) interfere with the binding site (Steinitz *et al.*, *Journal of Immunology* **141**: 3516-3522, 1988).

Furthermore, the idotypic network theory postulated that each Ab2 might serve as the antigen for an additional antibody (Ab3). This Ab3 antibody, like the original binding agent (Ab1) specifically binds to the adhesion molecule to which Ab1 specifically bound. Thus, in certain preferred embodiments of the first and second aspects of the invention, the immune response results in the generation of an antibody that specifically binds to the adhesion molecule.

In one non-limiting example, the injection of a selectin (*e.g.*, E-selectin) specific binding agent (Ab1) into an animal suffering from an immune condition, where the immune condition is inflammation, could inhibit leukocyte (*i.e.*, white blood cell) rolling on the endothelial cells lining the walls of a blood vessel through two different mechanisms. The first mechanism of this non-limiting example is dependent on the direct effect of Ab1 that will act to block the selectin on either the white blood cell surface or endothelial cell membrane, and make it unavailable for binding by a selectin ligand (*e.g.*, sialyl Lewis A)-bearing cell. The effect of Ab1 would be direct but unfortunately would also be short- term due to the rapid clearance of Ab1 from the circulation (Ab1 usually remained in the circulation for several hours or few days only). The second mechanism is dependent on the induction of anti-idiotypic (Ab2) and anti-anti-idiotypic (Ab3) Abs. Since Ab2 would mimic a selectin, it will inhibit through its binding to selectin molecules the interaction between selectins and their natural ligands. Ab3 will act as Ab1 and compete with the naturally existing carbohydrte selectin ligand in being bound by its selectin and as a result, inhibit the interaction between selectins and their ligands through competitive inhibition.

In another non-limiting example, the injection of a carbohydrate selectin ligand (*e.g.*, sialyl Lewis A) specific binding agent (Ab1) into an animal suffering from an immune condition, where the immune condition is cancer, could inhibit metastatic cancer cell (*e.g.*, melanoma cell) rolling on the endothelial cells lining the walls of a blood vessel through two different mechanisms. The first mechanism is dependent on the direct effect of Ab1 that will act to block selectin ligand on either white blood cell surface or endothelial cell membrane, and make it unavailable for binding by a selectin-bearing cell. The direct effect of Ab1

would be direct but short- term. The second mechanism is dependent on the induction of anti-idiotypic (Ab2) antibodies which mimic the selectin ligand and anti-anti-idiotypic (Ab3) antibodies which mimic the Ab1 antibody that specifically bound to the selectin ligand. Ab2 would mimic a selectin ligand, thus inhibiting cancer cell rolling on endothelial cells and extravasation through its binding to selectin molecules. Ab3 will inhibit cancer cell rolling and extravasaion by competing with the naturally existing selectin molecule in binding to carbohydrate selectin ligand.

The effect of Ab2 and Ab3 (*i.e.*, the indirect effect of Ab1) would likely to be more efficient than the direct effect of Ab1 for the inhibition of leukocyte or cancer cell migration, since Ab2 and Ab3 antibodies are produced by the treated animal and remain in the circulation for a considerable period of time (usually several months).

As used herein, the term "therapeutically effective amount" is used to denote a dosage of binding agent effective to reduce an immune condition in the treated animal and/or a dosage of binding agent effective to induce an immune response to the binding agent in the treated animal. Preferably, such administration should be intravenous, intra-arterial, subcutaneous, parenteral, or transdermal. Preferably, a binding agent of the invention is administered at a dosage of less than about 8 mg per 30 kg body weight of the animal to be treated; preferably less than about 5 mg per 30 kg body weight; preferably less than about 3 mg per 30 kg body weight; still more preferably from about 0.5 mg to about 2 mg per 30 kg body weight; still more preferably from about 0.5 mg to about 1.5 mg per 30 kg body weight; and most preferably, a binding agent of the invention is administered at a dosage of about 1 mg per 30 kg body weight of the animal to be treated.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that, when administered to an animal, does not induce antibody-dependent cellular cytotoxicity (ADCC) in the treated animal. In certain preferred embodiments of the first and second aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that is the maximum amount of binding agent that, when administered to an animal, does not induce antibody-dependent cellular cytotoxicity (ADCC) in the treated animal. In these embodiments, ADCC may be assessed by incubating ^{51}Cr-labeled tumor cells with a binding agent according to the invention and adding fresh human peripheral blood mononuclear cells (PBMCs), followed by incubation for four hours and then

measurement of specific lysis (as determined by ^{51}Cr release). ADCC is absent if specific lysis is less than 15%.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that, when administered to an animal, does not induce antibody-mediated toxicity in the treated animal. In certain preferred embodiments of the first and second aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that is the maximum amount of binding agent that, when administered to an animal, does not induce antibody-mediated toxicity in the treated animal. By "antibody-mediated toxicity" is meant clinical toxicity, such as abnormal serum chemistries, impaired renal function, signs and symptoms of serum sickness, or anaphylaxis. Antibody-mediated toxicity is well-known to immunologist and described, for example, in Abbas *et al.*, Cellular and Molecular Immunology, 3rd ed., W. B. Saunders Co., Philadelphia, PA 1997 (see particularly pages 425-434). In certain embodiments, a single such dosage will therapeutically treat the animal. In other embodiments, the treatment may be ongoing, *e.g.*, administration of a dosage of a binding agent four times a year for three or more years. In one example, the administration of a dosage of a binding agent will be performed one injection per month for about three months to years.

In accordance with the invention, the term, "blood-borne cell," means any cell that can be carried by the blood stream. Thus, the definition includes bone marrow derived cells such as red blood cells and white blood cells which include, without limitation, lymphocytes (both T and B), natural killer cells, neutrophils (also called polymorphonuclear leukocytes or PMNL), eosinophils, basophils, monocytes, megakaryocytes, and platelets. Also included as blood-borne cells are metastatic cancer cells which have migrated (or are attempting to migrate) from the site of the original abnormal cell.

Thus, in those embodiments of the first, second, third, and fourth aspects of the invention, where the immune condition is inflammation, the blood-borne cell is a white blood cell. In certain embodiments, the white blood cell is, without limitation, a lymphocyte, a platelet, a granulocyte (*i.e.*, a a neutrophil, eosinophil, or a basophil), or a monocyte.

In those embodiments of the first, second, third, and fourth aspects of the invention, where the immune condition is cancer, the blood-borne cell is a cancer cell. Any cancer cell that is carried by the blood stream is included in the invention. In certain embodiments, the

cancer cell is, without limitation, a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

As used herein, by "adhesion molecule" is meant a molecule which is expressed on the surface of or released by either a blood-borne cell or a cell (*e.g.*, an endothelial cell) that lines a blood vessel and which can form an association with another adhesion molecule expressed on the surface of either a blood-borne cell or a cell lining a blood vessel. By "released by" means an adhesion molecule that is either cleaved from the cell surface of the indicated cell, secreted by the indicated cell, or released from the indicated cell upon lysis of that cell. Preferably, an adhesion molecule of the invention is a molecule expressed on the surface of or released by either on a blood-borne cell or an endothelial cell lining a blood vessel that forms an association with a second adhesion molecule on a blood-borne cell or an endothelial cell lining a blood vessel, such that association formed is between a blood-borne cell-expressed or released adhesion molecule and an endothelial cell-expressed or released adhesion molecule. Preferably, an adhesion molecule of the invention is one that is involved in the extravasion of white blood cells or cancer cells from the blood into the surrounding tissue. Preferably, the definition of "adhesion molecule" excludes the B cell receptor, the T cell receptor, and/or the signalling molecules comprising the B cell receptor complex (*e.g.*, Ig-α) and the T cell receptor complex (*e.g.*, CD3). The site on an adhesion molecule of the invention which is specifically bound by the binding agent is called an "epitope."

Non-limiting examples of adhesion molecules include the molecules which are members of the integrin family of proteins including, without limitation, LFA-1 (CD11a/CD18), VLA-4 (CD49d/CD29), and Mac-1 (CD11b/CD18). Adhesion molecules also include those molecules which are members of the immunoglobulin gene family of proteins including, without limitation, ICAM-1, B7, CD4, and CD28. Adhesion molecules also include those molecules which are members of the selectin family of molecules including, without limitation, P-selectin ELAM-1, CD62E), E-selectin (ELAM-1, CD62E) and L-selectin (CD62L) (Bevilacqua and Nelson, *Journal of Clinical Investigation* **91**: 370-387, 1993. Thus, in certain embodiments of the first and second aspects of the invention, the adhesion molecule is a selectin, such as P-selectin, E-selectin, or L-selectin.

Adhesion molecules of the invention also include glycoproteins, such as PSGL-1 (P-selectin glycoprotein ligand 1) and molecules (*e.g.*, mucins or mucoproteins) comprising carbohydrate epitopes, including the Sialyl Lewis A (SLe(a)) carbohydrate epitope and the Sialyl Lewis X (SLe(x)) carbohydrate epitope. Thus, in certain embodiments of the first and

second aspects of the invention, the adhesion molecule of the invention is a molecule comprising a carbohydrate epitope, such as a molecule comprising a Sialyl Lewis A epitope. Preferably, a molecule comprising a carbohydrate epitope is specifically bound by a selectin molecule.

In various embodiments of the third and fourth aspects of the invention, the agent is a binding agent that specifically binds to an adhesion molecule.

As used herein, by "binding agent" is meant a molecule or macromolecule that specifically binds to an adhesion molecule either in water, under physiological conditions (*e.g.*, in an animal's body), or under conditions which approximate physiological conditions with respect to ionic strength, *e.g.*, 140 mM NaCl, 5 mM $MgCl_2$. By "specifically binds" is meant a binding agent of the invention recognizes and forms a covalent association or, preferably, a non-covalent association with an adhesion molecule of the invention, but does not substantially recognize and form a covalent or non-covalent association with other molecules in a sample, *e.g.*, proteins that are not adhesion molecules. Likewise, an adhesion molecule of the invention bound by a binding agent of the invention that specifically binds to that adhesion molecule is said to be "specifically bound" by that binding agent. Preferably, a binding agent of the invention that specifically binds to an adhesion molecule of the invention forms an association with that adhesion molecule with an affinity of at least $10^6 M^{-1}$, more preferably, at least $10^7 M^{-1}$, even more preferably, at least $10^8 M^{-1}$, and most preferably, at least $10^9 M^{-1}$.

In certain embodiments, a binding agent of the invention is a peptide, a peptidomimetic, or a carbohydrate. By "peptide" is meant a molecule comprised of a linear array of two or more amino acid residues connected to each other in the linear array by peptide bonds. By "peptidomimetic" is meant a non-peptide molecule that mimics the structure and/or function of a peptide. By "carbohydrate or oligosaccharide" is meant a molecule comprising sugar residues. Non-limiting binding agents that are carbohydrates include SLe(x) oligosaccharide (Buerke *et al.*, *Journal of Clinical Investigation* **93**: 1140-1148, 1994; Lefer *et al.*, *Circulation* **90**: 2390-2401, 1994), and SLe(a) oligosaccharide.

Non-limiting binding agents of the invention include antibodies. Thus, in certain embodiments of the first, second, third, and fourth aspects of the invention, the binding agent is an antibody, such as a monoclonal antibody. In certain preferred embodiments, the binding agent is a murine monoclonal antibody.

For example, the monoclonal antibody of the invention, HB1 (also called ALT-4), is a murine IgM monoclonal antibody (mAb) secreted by the HB1 hybridoma cell line. HB1 specifically binds to the tumor marker, CA19-9, which comprises the carbohydrate epitope Sialyl Lewis A (Mukae *et al.*, *Am. Rev. Respir. Dis.* **148**(3): 744-751, 1993). Immunoassay analysis has revealed that HB1 also specifically binds to Sialyl Lewis A. HB1 hybridoma cells were maintained in standard media (RPMI-1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin, 10% v/v fetal bovine serum (FBS)).

Another non-limiting antibody of the invention is HB2 (also called ALT-3), a IgG3 murine monoclonal antibody secreted by the HB2 hybridoma cell line. HB2 specifically binds to the tumor marker CA19-9, and has also been shown to specifically bind to sialyl Lewis A. HB2 hybridoma cells were maintained in standard media.

Yet additional non-limiting binding agents that are antibodies include PB1.3, an anti-P selectin monoclonal antibody (Weiser *et al.*, *Shock* **5**(6): 402-407, 1996; Yamada *et al.*, *European Journal of Pharmacology* **346**: 222-224, 1998); ARP2-41, an anti-rat-P-selectin mAb (Tojo *et al.*, *Glycobiology* **6**(4): 463-9, 1996; Ohnishi *et al. Immunopharmacology* **34**: 161-170, 1996); BBIG-E5, an anti-E-selectin mAb (Altavilla *et al.*, *European Journal of Pharmacology* **270**: 45-51,1994); DREG-200, an anti-L-selectin mAb, (Ma *et al.*, *Circulation* **88**: 649-658, 1993); MEL-14, an anti-L-selectin mAb (MEL-14) (Pizcueta and Luscinskas, *American Journal of Pathology* **145**(2): 461-467, 1994); anti- SLe(x) mAb (Yoshinori *et al.*, *Journal of Pathology* **180**: 305-310, 1996); anti- SLe(x) mAb (anti-human and rat) (clone no. 2H5; commercially available from BD Pharmingen, San Jose, CA); anti- SLe(x) mAb (clone no. CSLEX; commercially available from Becton Dickinson Immunocytometry Systems, San Jose, CA); anti- SLe(x) mAb (clone no. CSLEX1; commercially available from Signet Laboratories, Inc., Dedham, MA); anti-PSGL-1 (CD162) antibodies (*e.g.*, commercially available from Biodesign Intl., Saco, ME (*e.g.*, clone no. 3E2.25.5(PL1) (Catalog No. P91090M) and clone no. 5D8.8.12 (PL2) (Catalog No. P91209M) and the various anti-SLe(a) mAb commercially available from Biodesign Intl., Saco, ME (*e.g.*. clone no. 241 (Catalog No. M37241B); clone no. 59 (95-330-07) (Catalog No. M37059M); clone no. 62 (Catalog No. M37062M); clone no. 67 (Catalog No. M37067M); clone no. 192 (Catalog No. M37192M); and clone no. 239 (Catalog No. M37239M)).

Yet additional non-limiting binding agents of the invention include the anti-human E-selectin and anti-human P-selectin antibodies commercially available from R & D Systems (Minneapolis, MN, USA).

In certain embodiments, the binding agents of the invention comprises a complementarity determining region (CDR) of an antibody or a T cell receptor that specifically binds to an adhesion molecule of the invention. By "complementarity determining region (CDR) of an antibody" is meant a portion of an antibody that specifically binds to an epitope, including any framework regions necessary for such binding, and which is preferably comprised of a subset of amino acid residues encoded by the immunoglobulin gene heavy chain V, D, and J regions, the immunoglobulin gene light chain V and J regions, and/or any combinations thereof. By "complementarity determining region (CDR) of a T cell receptor" is meant a portion of a T cell receptor that specifically binds to an epitope (*e.g.*, in context of the major histocompatibility complex), including any framework regions necessary for such binding, and which is preferably comprised of a subset of amino acid residues encoded by the T cell receptor β chain gene V, D, and J regions, the T cell receptor α chain gene V and J regions, and/or any combinations thereof. Preferably, the CDR of the invention is derived from a human or a murine antibody.

Given the numerous antibodies disclosed herein, one of skill in the art are enabled to make a variety of antibody derivatives, wherein such antibody derivatives are also included as binding agents of the invention. For example, Jones *et al.* (*Nature* **321**: 522-525, 1986) disclose replacing the CDRs of a human antibody with those from a murine antibody. Marx, J.L. (*Science* **229**(4712): 455-456, 1985) discusses chimeric antibodies having murine variable regions and human constant regions. Rodwell, J.D. (*Nature* **342**(6245): 99-100, 1989) discusses lower molecular weight recognition elements derived from antibody CDR information. Clackson, T.P. (*Br. J. Rheumatol.* **30 Suppl 2**:36-39, 1991) discusses genetically engineered monoclonal antibodies, including Fv fragment derivatives, single chain antibodies, fusion proteins, chimeric antibodies, and humanized rodent antibodies. Reichman *et al.* (*Nature* **332**: 323-327, 1988) discloses a human antibody on which rat hypervariable regions have been grafted. And Verhoeyen *et al.* (*Science* **239**: 1534-1536, 1988) teaches the grafting of a mouse antigen binding site onto a human antibody.

In addition, given the numerous antibodies disclosed herein, those of skill in the art are enabled to design and produce peptidomimetics having binding characteristics similar or superior to such complementarity determining regions, wherein such peptidomimetics are included as binding agents of the invention. Peptidomimetics are well known and described, for example, in Horwell, D.C., *Bioorg. Med. Chem.* **4**: 1573-1576, 1996; Liskamp *et al.*, *Recl.*

Trav. Chim. Pays-Bas. **1**: 113, 1994; Gante *et al.*, *Angew. Chem. Int. Ed. Engl.* **33**: 1699, 1994; and Seebach *et al.*, *Helv. Chim. Acta* **79**: 913, 1996.

The binding agents of the present invention are preferably purified binding agents. By "purified" is meant that a binding agent is at least 60%, preferably at least 75%, even more preferably at least 90%, and most preferably at least 95% free from contaminants, such as endotoxins.

In a non-limiting example, where the binding agents of the invention are monoclonal antibodies, to purify the antibodies (*e.g.*, HB1 and HB2), the antibodies were purified from ascites. For ascites preparation, adult mice (age 6 weeks) were primed by injecting 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid) into the peritoneum (i.p.). Two weeks later the mice were injected i.p., with 5 x 10^6 hybridoma cells (*e.g.*, HB1 or HB2) resuspended in 0.5 ml of PBS. Ascetic fluid was tapped from mice 2 to 3 times in due time, and the collected fluid was incubated for 1 hour at room temperature and transferred to 4°C overnight. The fluid was centrifuged at 2000 rpm for 15 minutes and the supernatant was stored at –20 °C.

The monoclonal antibodies were then purified and concentrated. As various methods for purifying and concentrating antibodies are known to those of skill in the art, the following purification methods are non-limiting examples of methods that can be used for purifying antibodies from ascites. It should be noted that these methods can also be used to purify and concentrate antibodies from the supernatant of hybridoma cells grown *in vitro* under standard tissue culture conditions.

For mannan binding protein (MBPP affinity chromatography purification, the ascetic fluid containing the IgM was dialyzed against binding buffer (10 Mm Tris, 1.25 M NaCl, 20 mM $CaCl_2$, and 0.02 % Sodium azide (NaN_3) at a pH of 7.4), after which the ascetic sample was diluted 1: 1 v/v with binding buffer, and filtered with 0.22 mm filter. The MBP column (commercially available from Pierce, Rockford, IL, USA) was prewashed with 2 column volume (20 ml) of elution buffer (10 mM Tris, 1.25 M NaCl, 2mM EDTA and 0.02% NaN_3), and then equilibrated with 4 column volume (40 ml) of binding buffer. The ascetic fluid (maximum 1.5 ml/ 5 ml of gel) was applied to the column, allowed to completely enter the gel, and incubated at 4 °C for 30 minutes. The column was washed with 9 column volumes of the binding buffer to remove the unbound proteins. The wash was monitored for the presence of proteins by measuring the absorbance at 280 nm, using the binding buffer as a reference. The column was removed from the cold and incubated with the elution buffer at room

temperature for 1 hour, after which the column was washed with the elution buffer, and minimum of 14 fractions were collected each with a volume of 3 ml. The elution of IgM was monitored for the presence of proteins by spectrophotometer at 280 nm using the elution buffer as a reference. At the completion of the purification the column was washed with 2 column volume of deionized water and then by 2 column volumes of 4 °C binding buffer and stored at 4 °C.

The euglobulin precipitation method is a non-chromatographic method used for the purification of both murine IgG_3 and IgM mAbs, which takes advantage of their euglobulin properties. This method was performed as essentially described by (Garcia-Gonzalez et al., *Journal of Immunological Methods* **111**: 17-23, 1988). Briefly, $CaCl_2$ was added to the ascetic fluid (final concentration, 25 mM) to generate fibrin formation. When the clot was formed, it was removed by paper filtration. Then the filtered ascetic fluid was dialyzed for 2 hours at 20 °C (IgG3), or for 15 hours at 4 °C (IgM) against 100x volume of demineralized water (pH 5.5). The ascetic fluid was centrifuged in a BECKMAN L8-55 ultracentrifuge at 22,000 x g for 30 minutes and the precipitate was recovered and suspended in 1M NaCl/0.1 M Tris-HCl pH 8. Dialysis and precipitation were repeated twice. Purified pellet with a high lipid content was mixed with 1.7 M NaCl and centrifuged for 3 hours at 27,000 x g. Lipid supernatant was discarded, the clarified mAbs solution was dialyzed against 0.1M Tris HCl, 1M NaCl pH 8, centrifuged at 22,000 x g for 30 minutes. The purified pellet was suspended in phosphate buffered saline (PBS).

For Protein A affinity chromatography purification, a 5 ml Protein A column (commercially available from Pierce, Rockford, IL, USA) was washed with 2 column volumes of elution buffer (0.1 mM Glycine, 1mM NaCl, 0.001 % tween 20, pH 4). Then the column was equilibrated with 5 column volumes of binding buffer (50 mM Tris-HCl, 1mM NaCl, 0.001 % tween 20, pH 8). The ascetic sample containing IgG_3 antibody was diluted 1:1 v/v with binding buffer and loaded to the column at a rate of 0.75 ml/ minute, then the column was washed with 50 ml of binding buffer, at a rate of 1.5 ml / minute. The collected fractions containing unbound proteins were checked by spectrophotometer at 280 nm, using the binding buffer as a reference. The column was washed with 15 ml of elution buffer. Then the eluted fractions were collected at a rate of 1 ml /minute and adjusted to a pH of 7 using 50 mM Tris-HCl. Elution of bound proteins was monitored by spectrophotometer at 280 nm, using the elution buffer as a reference.

For removal of endotoxins from purified immunoglobulins, a 10 ml Detoxi-Gel column (commercially available from Pierce, Rockford, IL, USA) was regenerated by washing the gel with 5 column volumes of 1% sodium deoxycholate. Then the column was washed with 3 column volumes of pyrogen-free water. The purified and concentrated immunoglobulin was loaded to the column, and then the sample was collected by gravity flow using PBS as elution buffer.

Before use, the ultrafilter YM-30 membrane (MW cutoff 30,000 Dalton) (commercially available from Fisher Scientific, Nepean, ON, Canada) was floated with distilled water for one hour, changing the water three times. Then the membrane was mounted in an ultrafiltration cell and rinsed with distilled water at 20 psi (3.7 atm) for at least 5 minutes. The samples of purified Igs were loaded in the cell, and the sample flow through the ultrafilter membrane was operated under a maximum pressure of 40 psi (4.7 atm). The antibody solution was concentrated approximately 10x and then washed at least three time using PBS.

The quantification of purified F5 IgM, 7C2C5C12 IgM and Flopc-21 IgG_3 mAbs was done by ELISA where plates were coated with goat anti-mouse Ig (commercially available from Sigma Chemical Co., St. Louis, MO, USA). Note that this ELISA was also used to detect the presence of either Ab2 antibody in a treated animal of the invention, or the presence of rat anti-mouse antibody, since all of the injected antibodies are murine antibodies injected into rats. A schematic diagram of this assay is shown on Figure 3.

The quantification of HB1 IgM and HB2 IgG_3 mAbs was done by ELISA where the plates were coated with CA19-9 antigen. Note that this ELISA was also used to detect the presence of Ab3 antibodies. A schematic diagram of this assay is shown on Figure 4.

For each tested mAb, an isotype matched control mAb was used as a standard. Clarified ascites containing the mouse IgG3k monoclonal antibody Flopc-21 was obtained from SIGMA Chemical Co. (St Louis, MO, USA). Control F5 antibody is murine IgM monoclonal antibody secreted by the F5 hybridoma cell line (commercially available from the American Type Culture Collection, Maryland, USA). Control 7C2C5C12 antibody is a murine IgM monoclonal antibody which specifically binds to *Trichinella spiralis* and which is secreted by the 7C2C5C12 hybridoma cell line (commercially available from the American Type Culture Collection, Maryland, USA). Both F5 hybridoma cells and 7C2C5C12 hybridoma cells were maintained in standard media (RPMI-1640 supplemented with 2 mM

L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin, 10% v/v fetal bovine serum (FBS)).

For the ELISA assay for determining the concentration or the binding activity of mAbs, microtiter strips were coated with 100 ml of PBS containing either 2.5 µg/ml of goat anti-mouse Ig (whole molecule), 75 U/ml of CA19-9 antigen (commercially available from Altarex Corp., Edmonton, Alberta, Canada), or 2.5 µg/ml of polyacrylamide-SLe(a) (commercially available from Altarex Corp., Edmonton, Alberta, Canada) and incubated overnight at 4 °C. The coating solution was discarded and 150 µl of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS) was added to each well in order to block the non-specific binding sites on the wells. After one hour of incubation at room temperature the plates were washed three times with PBST. 100 µl of binding buffer containing mAbs was added in different dilutions. All wells were incubated for 1 hour at room temperature, followed by three washings with PBST (PBS containing 0.1% Tween-20, pH 7.1)). Then 100 µl of binding buffer containing goat anti-mouse Ig (H+L) -HRP labeled (commercially available from Southern Biotechnology Associates Inc., Birmingham, AL, USA) was added to each well at a dilution of 1/5000 v/v, incubated for 1 hour, and followed by three washings with PBST. The activity of bound mAbs was determined by adding a 100 µl of ABTS (Peroxidase solution B and ABTS peroxidase substrate, 1:1 v/v dilution) to each well (ABTS Peroxidase solution and ABTS peroxidase substrate commercially available from LifeTechnologies GIBCO BRL (Burlington, Ontario). The optical density was measured at dual wavelength, 405 and 492 nm using an ELISA reader (Titertek multiskan Plus MK II).

An SDS-polyacrylamide gel electrophoresis was used to check the purity of each of the purified mAbs. Ab samples were diluted 1:4 v/v with SDS-PAGE sample buffer (0.5 M Tris-HCl at pH 6.8, 20% glycerol, 10% w/v SDS, 10% b- mercaptoethanol and 0.5 % bromphenol blue) and heated for 4 minutes at 95 °C. The Tris-HCL ready gel sandwich (10% acrylamide) (commercially available from BioRad, Hercules, CA, USA) was inserted into the Mini-PROTEAN II cell clamp assembly and aligned properly. Then the gel sandwich was attached to the inner core of the Mini-PROTEAN II cell (commercially available from BioRad). The upper and the lower buffer chambers were filled with approximately 200 ml of 1x running buffer (0.3% Tris base, 1.45% glycine, 0.1% SDS, pH 8.3). 6 µl of molecular weight markers in sample buffer was applied to one of the wells, while the antibody samples, each with a volume of 30 µl (1.5-5 µg of antibody) were applied

to the rest of the wells in the gel. The core assembly was inserted into the lower buffer tank and the gel was run at 200 V constant voltage for approximately 45 minutes. The gel was then removed from the gel sandwich, immersed in staining solution (10% acetic acid, 0.025% Coomassie BlueG-250, methanol 40%) for 1.5 hour, and destained in destaining solution (10% acetic acid, 40% methanol) until the desired destaining was reached.

Antibodies of the invention were purified and then resolved by SDS-polyacrylamide gel electrophoresis. As shown in Figure 5, antibodies purified by the mannan binding protein affinity chromatograph (lanes 2 and 4), by the euglobin precipitation method (lanes 3 and 7), and by protein A affinity chromatography (lane 6) are "purified" for the purposes of the present invention (compare to lanes 5 and 8 for old samples of purified monoclonal antibodies.

In the non-limiting embodiment where the binding agent of the invention is a monoclonal antibody, the binding of the various murine monoclonal antibodies to tumor antigen CA19-9 and to sialyl Lewis A was determined. Using the ELISA assay described above and schematically depicted in Figure 4 (using CA19-9 coated plates), the various purified monoclonal antibodies were tested for binding to purified CA19-9. A range of mAb concentration from 0 to 0.64 mg/ml was used. As can be seen in Figure 6, for both HB1 (ALT-4) and HB2 (ATL-3) mAbs, even at a concentration of only 10 ng/ml, maximum binding to CA19-9 was observed which is likely to be due to the presence of multiple sialyl Lewis A epitopes within the CA19-9 antigen. As expected, the two control mAbs F5 and 7C2C5C12 did not bind to CA19-9 antigen. Surprisingly, however, the control mAb Flopc-21 that was used in the same concentration range bound to CA19-9 with an observed dose effect.

In order to determine more specifically the epitope recognized by HB1 and HB2, a second ELISA assay (as described above) was performed which used the carbohydrate antigen SLe(a) conjugated to polyacrylamide in order to carry a direct binding assay. As shown in Figure 7, both HB1 (ALT-4) and HB2 (ALT-3) mAbs recognized the carbohydrate epitope SLe(a). In addition, Figure 7 demonstrated that Flopc-21 mAb is directed against a different CA19-9 epitope determinant than the SLe(a) epitope recognized by HB2 and HB1 and, thus, does not interact with SLe(a) carbohydrate oligosaccharide.

The specificity of HB1 mAb for SLe(a) was also studied. To do this, HB1 was first radiolabeled with ^{125}I (iodinated). 50 μg of HB1 mAb was mixed with approximately 600

μCi of Na ^{125}I. 10 ml of 2.5 mg/ml of chloramine-T (CT) (commercially available from Sigma Chemical Co.) was added to the reaction vial and gently shaken for 30 seconds. Then, 10 μl of 5 mg/ml sodium metabisulfate (Na-Met) was added to the mixture and shaken gently for 15 seconds. Finally, 20 μl of 1M sodium iodide (NaI) was added and the whole mixture was gently shaken for 5 seconds. The reaction mixture was loaded to the Desalting Econo-pac 10 DG chromatographic column (commercially available from BioRad, Hercules, CA, USA) pre-washed three times with 0.5% BSA in PBS. The vial was rinsed with 100 μl of PBS and transferred to the column. The column was then washed with 0.5 ml PBS fractions 12 to 14 times. Each elute was collected separately in numbered tubes. The radioactivity of elutes was monitored by a Geiger counter. The fractions containing the mAbs were pooled, labeled, placed in lead containers, and stored at 4 °C. The quantification of ^{125}I labeled HB1 mAb in the pooled fractions was done by ELISA as described above (and depicted in Figure 4) using plates coated with CA19-9. A nonradioactive control HB1 mAb was used as standard.

For competitive inhibition assay, RIA strips coated with 50 μl of PBS containing a 65 U/ml of tumor antigen CA19-9 were incubated overnight at 4 °C. After discarding the coating solution, the non-specific binding sites were blocked with 150 μl/well of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS). The wells were incubated for 1 hour at room temperature, and then washed with PBST three times. Dilutions of SLe(a) antigen (commercially available from Oxford GlycoSciences Inc., MA, USA) were made serially in binding buffer (1% BSA, 0.02% thimerosal in PBS) from 0 to 100 μg / ml and mixed with iodinated HB1 mAb. A nonsaturating concentration (0.33 μg/ml) of iodinated HB1 (ALT-4) mAb was chosen in order to obtain a final count per minute (c.p.m.) concentration of approximately 40,000 c.p.m./well. 100 μl of each dilution was added to different wells and allowed to incubate for 1.5 hours. After that the supernatant was discarded, the strips were washed with PBST three times and broken down to individual wells and the radioactivity was measured (bound fraction).

As shown in Figure 8, only at high concentrations, namely 50 μg /ml and 100 μg/ml, was SLe(a) antigen able by competitive inhibition to reduce the binding of HB1 to CA19-9 antigen by 35% and 60% respectively. The requirement for such a high concentration of Sle^a to inhibit the binding of HB1 mAb to CA19-9 could be due to the fact that HB1 Ab is an IgM and therefore has 10 binding sites for each SLe(a) molecule. In addition, the affinity of HB1

Ab for the SLe(a) epitope carried by CA19-9 is likely to be higher than the affinity of HB1 Ab for soluble SLe(a). Such difference in affinity may be due to a more stabilized three-dimensional configuration of the SLe(a) carried by CA19-9 and/or to a longer sequence recognized by HB1 within CA19-9 molecule.

The affinity of HB1 (ALT-4) mAb to Ca19-9 antibody was next determined by scatchard analysis using iodinated HB1 mAb. To do this, radio-immunoassay (RIA) strips were coated with 100 μl of PBS containing 65 U/ml of CA19-9 antigen overnight at 4 °C. After discarding the coating solution, the nonspecific binding sites were blocked with 150 μl/well of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS). The wells were incubated for 1 hour at room temperature with gentle shaking, and then washed three times with PBST (PBS, 0.1% Tween-20, pH 7.1). Each strip was then incubated with a range of concentration from 0.005 to 10.5 μg/ml of I^{125} labeled HB1 antibody in 100 μl of binding buffer (1% BSA, 0.02% thimerosal in PBS) at room temperature for 1 hour. Then, 50 μl of the HB1 supernatant was taken from each well and the radioactivity was measured (free fraction) gamma counter. The strips were again washed three times with PBST, broken into individual wells and the radioactivity of each well was measured (bound fraction) by Gamma counter.

Thus, the affinity of iodinated HB1 antibody to CA19-9 antigen was determined by measuring the radioactivity of free and bound fractions in individual wells by Gamma counter. As shown in Figure 9, the Kd value from this experiment was calculated to be equal to 0.94 nM.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

Example I

Male Sprague Dawley (SD) rats age 6 weeks, weight 275-300 grams, were injected five times with 250 mg/rat of either keyhole limpet hemocyanin (KLH)-conjugated HB1 mAb, control KLH-conjugated F5 mAb, or were simply received PBS injection via intraperitoneal (i.p.) route.

Serum collection was routinely done prior to each immunization. The tails of rats were cut slightly at the tip and the blood was collected in serum separator microtainers. During this procedure the rats were anaesthetized with ethyl ether. The collected blood was centrifuged at 300 x g for five minutes; serum was separated, and used to test for the detection of RAMA and Ab3 responses. The animals were kept under care until inflammation was induced and measured.

The mAbs were conjugated to KLH in order to enhance their immunogenicity. Chemical conjugation is usually very efficient but is likely to decrease the binding activity of the mAbs. For each conjugation, we therefore determined the remaining binding activity of the KLH-mAbs. This was done by direct ELISA as described above and schematically depicted in Figure 4 using CA19-9 coated plates.

To conjugate antibodies with keyhole limpet hemocyanin (KLH), 1 mg of either HB1 mAb or F5 mAb (control) was suspended in PBS solution was mixed with 1 mg of KLH (commercially available from SIGMA Chemical Co., St Louis, MO, USA) and 2 ml of glutaraldehyde (final concentration, 25%). The mixture was incubated for 1 hour at room temperature and then 75 mg of glycine was added to the mixture. The mixture was stirred for an addition 1 hour and then dialyzed against distilled water for three days. The binding activity of the mAbs to CA19-9 antigen was measured before and after conjugation with KLH and compared by ELISA using CA19-9 antigen-coated plates.

Rat anti-mouse antibody (RAMA) and Ab3 responses were measured between each injection. The ELISA assays used for the measurement of Ab2 (RAMA) and Ab3 humoral responses are described above and illustrated in Figures 3 and 4, respectively.

The rats were then subjected to acute inflammation in the right hind paw by injection of carrageenan (carrageenan lambda type IV (gelatin, vegetable, Irish moss); commercially available from Sigma Chemical Co.) five days after the last injection of antibody. To induce inflammation in the rat right hind paw (intraplantary), 0.05 ml of 1% carrageenan in 0.9 % NaCl as injected into the rats five days after the last immunization with antibody (*e.g.*, KLH conjugated HB1, F5, or PBS only). The animals were lightly anaesthetized with ethyl ether for the injections and for inflammation measurements.

Paw edema was used as a characteristic sign of inflammation, and was quantitated at regular interval of time after carrageenan injection, with a total of 8 measurements. Paw edema was measured by two different methods. The caliper method was used to measure

paw thickness, and water displacement method to measure paw volume. The thickness of rat paw was measured in inches by a caliper device before and after carrageenan injection at desired time intervals. The paw volume was measured by immersing the paw up to the tibio-tarsae articulation in a cylinder filled with water, and the fluid volume replaced by immersed paw was measured before and after carrageenan injection at desired time intervals.

Both paw thickness and paw volume measurements of the inflammatory response results were analyzed in the same way. The measurements were plotted as area under the curve (AUC) percent change in effect and as percent change in maximum effect. "Percent change in effect" means the percent change in inflammatory response in each rat from time zero to each consecutive time of inflammation measurement. "AUC percent change in effect (area under the curve)" means the sum of the percent changes in inflammatory response from time zero to each consecutive time of inflammation measurement for each rat. The average of AUC percent change in effect was calculated per rat group. "Percent change in maximum effect" means the percent change in inflammatory response from time zero to the time where maximum inflammation has occurred in each rat.

Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 10A, 10B, and 10C; and as percent change in maximum effect in Figures 11A, 11B, and 11C. Paw volume measurement results are illustrated as AUC percent change in effect in figures 12A, 12B, and 12C; and as percent change in maximum effect in Figures 13A, 13B, and 13C.

For both paw thickness and paw volume measurements, the least edema was observed in HB1 group, but this difference in change in effect, measured by caliper method (P= 0.631) or by water displacement method (P= 0.655), was not statistically significant.

Ab3 was not detected in any of the rat groups tested. The results obtained for the measurement of the RAMA immune response are shown in Figure 14. The RAMA response was detected in only one rat in the group of rats treated with HB1 mAb (rat # 5, Figure 15). The PBS only-injected group did not receive any mAb injection and consequently had no RAMA response (Figure 14). The absorbance obtained by ELISA corresponds therefore to background level (Figure 14). The F5 control mAb-injected group showed absorbance values above background level. These values correspond to anti-isotypic and/or anti-allotypic immune responses (the assay performed with HB1 coated plates cannot measure the anti-idiotypic response specific for F5 antibody). The HB1 therapeutic mAb-injected group

showed absorbance values above background level that corresponds to either anti-isotypic, anti-idiotypic or anti-allotypic immune response or a combination of them, among which only the idiotypic response will have the therapeutic effect.

It is worth noting that in the HB1 group, one rat (rat # 5) had a high RAMA response (see Fig. 14). Of particular interest, this particular rat did not develop any inflammatory response (see Figures 10B and 12B).

Example II

Male Sprague Dawley rats (age six weeks) were injected four times with 500 μg/rat of either KLH-conjugated HB1, control KLH-conjugated F5, control KLH-conjugated 7C2C5C12 mAbs, or have simply received PBS injection via i.p. route.

RAMA and Ab3 responses were measured between each injection. Induction and measurement of inflammation were done by the same methods as described in Example I. Paw edema was observed and measured at intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as described in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figure 15A, 15B, and 15C; and as percent change in maximum effect in Figures 16A, 16B, and 16C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 17A, 17B, and 17C; and percent change in maximum effect in Figures 18A, 18B, and 18C.

For both paw thickness and paw volume measurements, the least edema was observed in HB1-injected group, but this difference in change in effect, measured by caliper method ($P= 0.134$) or by water displacement methods (0.073) was not statistically significant.

Ab3 was not detected in any of the rat groups tested. A very low RAMA immune response was detected in the control groups (F5 and 7C2C5C12). The HB1-injected group was determined using the method depicted in Figure 3 to be the only group with a high RAMA immune response (Figure 19). Despite the fact that the group treated with HB1 antibody had the highest immune response, no significant correlation was found between the RAMA response and the therapeutic efficacy as shown in Figure 19.

The inflammatory responses of the HB1 injected rats at different measurement times was potted as percent change in effect against the RAMA (Ab2) response. As shown in

Figures 20A-20H, the correlation between the therapeutic efficacy and RAMA in HB1 group was not found to be significant at any time of the inflammation measurements.

Example III

Male Sprague Dawley rats (age six weeks) were injected four times with 500 mg/rat of either HB1, control F5 mAbs mixed with QUIL A adjuvant (commercially available from Calbiochem, San Deigo, USA), or have simply received PBS injection via subcutaneous (s.c.) route. RAMA and Ab3 responses were measured between each injection at regular intervals. Induction and measurement of inflammation was done by the same methods as described in Example I. Paw edema was observed and measured at regular intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 21A, 21B, and 21C; and as percent change in maximum effect in Figures 22A, 22B, and 22C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 23A, 23B, and 23C; and as percent change in maximum effect in Figures 24A, 24B, and 24C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method ($P= 0.253$) or by water displacement method ($P= 0.358$) was not statistically significant.

Ab3 was not detected in any of the rat groups tested. RAMA immune response was detected in the therapeutic HB1 group, and to a lesser extent in the control F5 group as shown in Figure 25 using the method as depicted in Figure 3.

The extent of inflammatory response was correlated with the extent of the level of RAMA immune response in HB1 group as shown in Figures 26A-26G. Of particular interest is the demonstration in HB1 rat group of a good correlation between RAMA immune response and the inflammatory response, *i.e.* the higher the RAMA immune response, the lower the inflammatory response. Surprisingly, some correlation was also observed between the extent of the inflammatory response and the RAMA response in the group treated with F5 mAb as shown in Figures 27A- 27G.

Example IV

Male Sprague Dawley rats (age six weeks) were injected four times with 500 μg/rat of either HB1 mAb or have simply received PBS injection via intravenous (i.v.) route. RAMA and Ab3 responses were measured between each injection at regular intervals. Induction and measurement of inflammation was done by the same methods as in experiment 1. Paw edema was observed and measured at regular intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 28A, 28B, and 28C; and as percent change in maximum effect in Figures 29A, 29B, and 29C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 30A, 30B, and 30C; and as percent change in maximum effect in Figures 31A, 31B, and 31C.

For both paw thickness and paw volume measurements, the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method (P= 0.111) or by water displacement method (P= 0.188) was not statistically significant.

Ab3 was not detected in any of the rat groups tested.

As shown in Figure 32, the RAMA immune response was detected (using the method depicted in Figure 3) in one rat that belonged to the therapeutic HB1 group. AUC percent change in effect from the measurements of caliper and water displacement methods showed that one rat in the therapeutic HB1 group had responded with minimal inflammatory response as shown in Figures 28B and 30B, respectively. Therefore, the extent of the inflammatory response as shown in Figures 28B and 30B was compared to the level of RAMA immune response in each rat in Figure 32. In fact, the same rat that showed the highest RAMA immune response as shown in Figure 32 also showed the least inflammatory response measured by both caliper and water displacement methods as shown in Figures 28B and 30B, respectively.

Example V

Rats were injected four times with 500 mg/rat of either KLH-conjugated HB2, control KLH-conjugated Flopc-21 mAbs, or have simply received PBS injection via i.p. route. RAMA and Ab3 immune responses were measured between each injection. Induction and

measurement of inflammation were done by the same methods as described in Example I. Paw edema was observed and measured at regular intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as described in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 33A, 33B, and 33C; and as percent change in maximum effect in Figures 34A, 34B, and 34C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 35A, 35B, and 35C; and as percent change in maximum effect in Figures 36A, 36B, and 36C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method (P=0.074) or by water displacement method (P= 0.065) was not statistically significant.

Ab3 was not detected in any of the rat groups tested in Example V. Using the method depicted in Figure 3, all tested groups showed minimal (background) RAMA immune response as shown in Figure 37.

Example VI

The binding of HB1 (ALT-4), HB2 (ALT-3), and control monoclonal antibodies F5 (IgM), 7C2C5C12 (IgM), and Flopc-21 (IgG3) to PMNLs and lymphocytes was quantitated by FACScan and fluorescent microscopy. The binding of HB1, HB2, and control mAbs to HUV-EC-C cells was also examined by fluorescent microscopy.

Human umbilical vein endothelial cells (HUV-EC-C) is an endothelial cell line derived from the vein of a normal, human umbilical cord, obtained from American Type Culture Collection (Maryland, USA). HUV-EC-C cells were maintained in MCDB 131 media supplemented with 10 ng/ ml of HEGF, 12 mg/ ml of BBE, 1 mg/ ml of hydrocortisone, 5 % v/v FBS, 0.05 mg/ ml of Gentamycin sulfate, and 0.05 mg/ ml of amphotericin B.

To isolate human white blood cells, 6 ml of Histopaque –Ficoll solution was added to a 15 ml test tube, and then a 6-ml of heparin-treated fresh human blood was carefully layered on top of the gradient. Tubes were centrifuged at 1700 rpm (450 x g) for 35 minutes at room temperature. The band at the upper interface (mononuclear cells) was retrieved (5 ml) and mixed with 10 ml of 0.45% NaCl and 20 ml of PBS (8 mM Di-sodium hydrogen

orthophosphate, 3 mM potassium dihydrogen orthophosphate, 0.14 M NaCl). The tubes were centrifuged at 1500 rpm (400 g) for 15 minutes at 21 °C. The pellet was retrieved and washed three times with 10 ml of 3 mM EDTA, 1% FBS in PBS solution without magnesium and without calcium and centrifuged at 800 rpm (250 x g) for 10 minutes. The pellet was suspended in 2 ml of RPMI + 5% fetal bovine serum (FBS). In the case of isolating human PMNL, the band at the lower interface was retrieved and treated in the same way as with monouclear cells. The purified cell pellet (mononuclear or PMNL cells) was suspended at 1x 10^7 cells/ml in RPMI media, 5% FBS and kept at 37 °C until used. Cell viability was determined by exclusion of 0.4% trypan blue.

For FACS analysis, 1x10^6 white blood cells or HUV-EC-C washed in PBS, 1% bovine serum albumin (BSA), were incubated with primary antibody (*i.e.*, HB1, HB2, F5, 7C2C5C12, or Floppc-21) at a concentration of 5 μg/ ml in PBS, 1% BSA. The incubation was performed at 4 °C and after 45 minutes the cells were washed three times with PBS, 1% BSA. The cells were then incubated with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse antibody at 4 °C for 45 minutes and washed three times. The stained cells were resuspended in PBS, 1% BSA. Fluorescence associated with the cells was analyzed either by FACScan flow cytometry (Becton Dickinson, Mountain View, CA) or by fluorescence microscopy. As negative control cells were incubated according to the same procedure but without primary antibody.

Examples of histograms obtained by FACS analysis are shown in Figures 38A-38H. Colo 205 cells, which were used as a positive control (Figs. 38A and 38B), are known to express the Sialyl Lewis A epitope on their surface. Colo 205 cells are a human adenocarcinoma cell line derived from metastatic colon cancer that produces CA19-9 antigen. Colo 205 cells were obtained from the American Type Culture Collection (Maryland, USA), and were maintained in standard media (RPMI-1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin, 10% v/v fetal bovine serum (FBS)).

As can be seen in Figures 38C-38H, all tested mAbs did not show significant binding to human neutrophils. None of the antibodies tested showed significant binding to rat neutrophils nor to HUVEC cells.

Example VII

The *in vitro* inhibition of leukocyte rolling on microchips by the therapeutic antibodies of the invention was next determined. For these studies, a microchip glass wafer device was used that consists of channels in which in vitro cell rolling and adhesion can be observed under flow conditions, the process that mimics the *in vivo* cell rolling and adhesion events.

To investigate the rolling and adhesion of cells, a 10 cm x 10 cm glass wafer with a series of simple "Y" mixer flow manifolds was used in this assay. The "Y" mixer flow manifold consisted of 50 microns in depth and 300 microns in width channels with a flow region downstream. These channels are structured in similar way to capillaries and therefore, the use of such channels allows the observation of cell rolling and adhesion in vitro that mimics the *in vivo* cell rolling and adhesion events. The microchip was secured into an aluminum microchip holder, and the microchip reservoirs were glued onto the microchip with 5-minute epoxy resin. The channels were conditioned with concentrated nitric acid (HNO_3), 2 M of sulfuric acid (H_2SO_4) and 1 M of sodium hydroxide (NaOH) (10 minutes each) before and after each experiment. The channels were then coated with 20 μg/ml of recombinant human E-selectin or P-selectin (commercially available from R & D Systems, Minneapolis, MN, USA) for 2 hours at 37 °C to mimic the surface of a vessel wall endothelial lining. A microsyringe pump that operated as a negative pressure source was used to drive the cells through the channels at a flow rate of 2.5 ml/min for the 50 micron deep device. This flow rate ensured a high enough shear rate (800 s^{-1}) to mimic blood flow in a vascular capillary. The channels were monitored using a Reichert Microstar (25X 280 x 220 mm field of view) and JVC color CCD camera mounted on the microscope (see Figure 39A). The experiments were recorded onto tapes with a JV VHS recorder.

A 1x 10^7 cells/ml solution of human PMNL or lymphocytes (isolated as described in Example VI) suspended in RPMI, 5% FBS was pumped to the channel through an injection route and allowed to run at an optimum flow rate for the purpose of achieving the rolling step. Since each channel had two sides, one side of each channel was used to test the agent under study and the other side of the channel was used as a control for cell rolling only without the addition of any agents (see Figure 39B). The cell suspensions added to different sides of the channel were regulated by a luminal fluidic flow that allowed these cell suspensions to continue running at different sides of the channel and prevented cell diffusion from one side of the channel to the other.

The following agents were tested at a volume of 10 µl and at a dilution of 1:10 v/v in RPMI and 5% FBS: fresh human serum (negative control), fresh rat serum, HB1 rat serum; and F5 rat serum. 10 µl of 100 µg/ml of anti-human E-selectin antibody (commercially available from R & D Systems, Minneapolis, MN, USA) was used as positive control for E-selectin coated channels. Serum of rats immunized with HB1 monoclonal antibody from Example III was obtained from rats who showed a high RAMA response (rat # 2, 4, 5, 6, 7, and 8, collectively). Serum of rats immunized with F5 monoclonal antibody from Example III was obtained from rats who showed a high RAMA response (rat # 4, 6, and 7, collectively). Once the rolling step was achieved, 10 µl of each of the above mentioned agent was added to different channels. The pump sucked the added sample into the channel to start the inhibition step. The images of cell rolling and inhibition steps were captured at different times and the number of cells was counted in both sides of the channel. Non-adherent cells appeared as streaks on the video image. The cell counts were conducted on a PC computer installed with a perception video board and image Pro Plus software.

The same agents were also tested on P-selectin coated channels under the same conditions as with E-selectin coated channels. For P-selectin coated channels, anti-human P-selectin antibody (commercially available from R & D Systems, Minneapolis, MN, USA) was used as a positive control.

Since there was no consistent rolling of rat leukocytes on the recombinant human E- or P-selectin coated channels and since rat E- and P-selectins were not available, the assay was carried only with human leukocytes (and not with rat leukocytes). The images of cell rolling and inhibition steps were captured at different times and the rolling cells were counted on both sides of the channel (see Figures 39A and 39B). The effect of each of the tested agents was determined as the percentage of rolling human PMNL from those in the control channel (100%) in which no agents were added.

As shown in Figures 40 and 41, respectively, normal human serum did not cause a reduction in human PMNL rolling on human E-selectin or human P-selectin coated channels. The same experiment performed with normal rat serum instead of normal human serum revealed by microscopic observation that such treatment resulted in an increased adherence of a small number of leukocytes on both E- and P- selectin coated channels. This effect however was very mild and did not affect the rolling of cells on either E- or P- selectin coated channels as shown in Figures 40 and 41, respectively.

Anti-E- and anti P-selectin mAbs were used to demonstrate effective rolling inhibition of PMNL on E-selectin (Fig. 40) and P- selectin (Fig. 41) coated channels, respectively. There was only 1.4 % of rolling cells observed with the addition of anti-E- selectin mAb, as compared to the side with the control rolling cells as shown in Figure 40, and 14.4% rolling cells with the addition of anti-P-selectin mAb as shown in Figure 41. Therefore, the rolling of human PMNL leukocytes on recombinant human E- and P- selectin coated channel was inhibited significantly by anti-E- and anti-P- selectin mAbs ("+ve control" in Figs. 40 and 41).

It was of particular importance to observe that the addition of 1:10 dilution of the RAMA positive serum obtained from rats immunized with HB1 antibody (rat # 2, 4, 5, 6, 7 and 8 collectively, Example III) caused a significant inhibitory effect on PMNL rolling on both human E-selectin and human P- selectin coated channels ("ALT-4 rat serum Ab2 +" in Figs. 41 and 42, respectively). In fact, the HB1 rat serum (*i.e.*, the ALT-4 rat serum from rats who showed a positive RAMA response) was able to reduce the rolling of PMNL on E-selectin coated channels to 0% resulting in a 100 % inhibition (P< 0.001) (see Fig. 40). The same rat serum resulted in a 76 % inhibitory effect on P-selectin coated channels (P< 0.001), since it was able to reduce the rolling of PMNL on P-selectin coated channels from 100 % to 23.2 % (see Fig. 41). In opposition to normal serum, no change in cell adherence or cell morphology was observed with the addition of rat serum immunized with HB1 antibody.

Unexpectedly, the RAMA positive control F5 rat serum also showed some inhibition of the rolling of PMNL leukocytes on recombinant human E- and P-selectin coated channels ("F5 rat serum Ab2+" in Figs. 40 and 41). However, this inhibition was considerably less marked than the one observed with the therapeutic monoclonal antibody HB1. The addition of serum obtained from rats immunized with F5 antibody who were positive for RAMA response (rats # 4, 6 and 7 collectively, Example III) caused a 45 % reduction in PMNL rolling on E-selectin coated channels (P< 0.001); and a 33 % reduction in PMNL rolling on P-selectin coated channels (P< 0.001) as shown in Figures 40 and 41 respectively.

Example VIII

A rodent model of melanoma is used to determine the effect of the binding agents of the invention in reducing melanoma. A murine melanoma cell line, such as clone B16 (commercially available as Catalog No. CRL-6322 from the American Type Culture

Collection, Manassas, VA) is injected subcutaneously or intra-peritoneally into syngeneic mice (*e.g.*, C57BL/6J mice) or immunodeficient nude mice (both commercially available from The Jackson Laboratory, Bar Harbor, ME) at an established primary site in an amount sufficient for the melanoma cells to establish tumor growth at the primary tumor site in the mice. A first set of mice is injected, and the time needed for metastasis of the melanoma cells to occur is recorded.

The experimental set of rats is injected with the melanoma cells. At the time metastasis is known to occur based on the results obtained from the first set of mice, the experimental set is divided into different groups of animals, each of which is administered a therapeutically effective amount of the various binding agents of the invention including, without limitation, HB1, HB2, ARP2-41, DREG-200, MEL-14, BBIG-E5, and anti-sialyl Lewis X antibodies via a subcutaneous, intraperitoneal, or intravenous injection. Control antibodies include 7C2C5C12 and F5.

Mice injected with binding agents of the invention that specifically bind to adhesion molecules expressed either on the cell surface of the melanoma cells or on the cell surface of endothelial cells lining blood vessel walls are found to have a reduced number of tumor formation at secondary sites (*i.e.*, sites distinct from the primary tumor site) than those mice injected with the control antibodies.

CLAIMS

1. A method for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition, comprising administering to the animal a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent.

2. The method of claim 1, wherein the adhesion molecule is a molecule comprising a carbohydrate epitope.

3. The method of claim 2, wherein the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A.

4. The method of claim 1, wherein the adhesion molecule is a selectin.

5. The method of claim 4, wherein the selectin is P-selectin or E-selectin.

6. The method of claim 1, wherein the immune condition is inflammation.

7. The method of claim 6, wherein the blood-borne cell is a white blood cell.

8. The method of claim 7, wherein the white blood cell is selected from the group consisting of a lymphocyte, a platelet, a granulocyte, and a monocyte.

9. The method of claim 1, wherein the immune condition is cancer.

10. The method of claim 9, wherein the blood-borne cell is a cancer cell.

11. The method of claim 10, wherein the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

12. The method of claim 1, wherein the binding agent is an antibody.

13. The method of claim 12, wherein the antibody is a monoclonal antibody.

14. The method of claim 1, wherein the immune response results in the generation of an antibody that specifically binds to the binding agent.

15. The method of claim 1, wherein the immune response results in the generation of an antibody that specifically binds to the adhesion molecule.

16. The method of claim 1, wherein the immune response comprises a humoral and a cellular immune response.

17. The method of claim 1, wherein the animal is a domesticated animal.

18. The method of claim 1, wherein the animal is a human.

19. The method of claim 1, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal.

20. The method of claim 1, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal.

21. The method of claim 1, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

22. A composition for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition comprising a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent.

23. The composition of claim 22, wherein the adhesion molecule is a molecule comprising a carbohydrate epitope.

24. The composition of claim 23, wherein the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A.

25. The composition of claim 22, wherein the adhesion molecule is a selectin.

26. The composition of claim 20, wherein the selectin is P-selectin or E-selectin.

27. The composition of claim 22, wherein the immune condition is inflammation.

28. The composition of claim 28, wherein the blood-borne cell is a white blood cell.

29. The composition of claim 28, wherein the white blood cell is selected from the group consisting of a lymphocyte, a platelet, a granulocyte, and a monocyte.

30. The composition of claim 22, wherein the immune condition is cancer.

31. The composition of claim 30, wherein the blood-borne cell is a cancer cell.

32. The composition of claim 31, wherein the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

33. The composition of claim 22, wherein the binding agent is an antibody.

34. The composition of claim 33, wherein the antibody is a monoclonal antibody.

35. The composition of claim 22, wherein the immune response results in the generation of an antibody that specifically binds to the binding agent.

36. The composition of claim 22, wherein the immune response results in the generation of an antibody that specifically binds to the adhesion molecule.

37. The composition of claim 22, wherein the immune response comprises a humoral and a cellular immune response.

38. The composition of claim 22, wherein the animal is a domesticated animal.

39. The composition of claim 22, wherein the animal is a human.

40. The composition of claim 22, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal.

41. The composition of claim 22, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal.

42. The composition of claim 22, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

43. The composition of claim 22, further comprising a pharmaceutically-acceptable carrier.

44. A therapeutic composition comprising an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount.

45. The composition of claim 44, wherein the agent is a binding agent that specifically binds to an adhesion molecule.

46. A method for reducing an immune condition in an animal suffering from cancer or inflammation or predisposed to suffer from cancer and inflammation, comprising administering to the animal a therapeutically effective amount of an agent that is capable of reducing inflammation and reducing cancer in an animal

47. The method of claim 46, wherein the agent is a binding agent that specifically binds to an adhesion molecule.

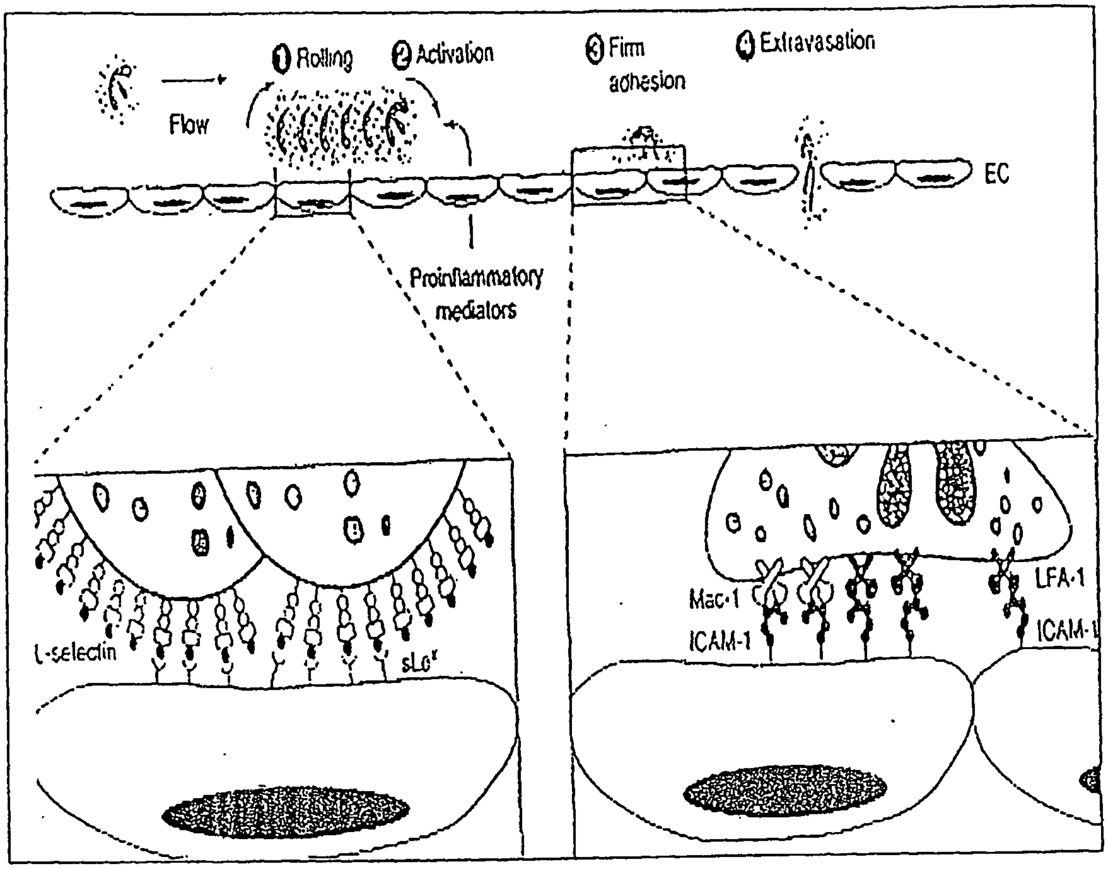

Rolling
Activation
Firm adhesion
Extravasation
Flow
EC
Proinflammatory mediators
L-selectin
sLe^x
Mac-1
ICAM-1
LFA-1

Figure 1

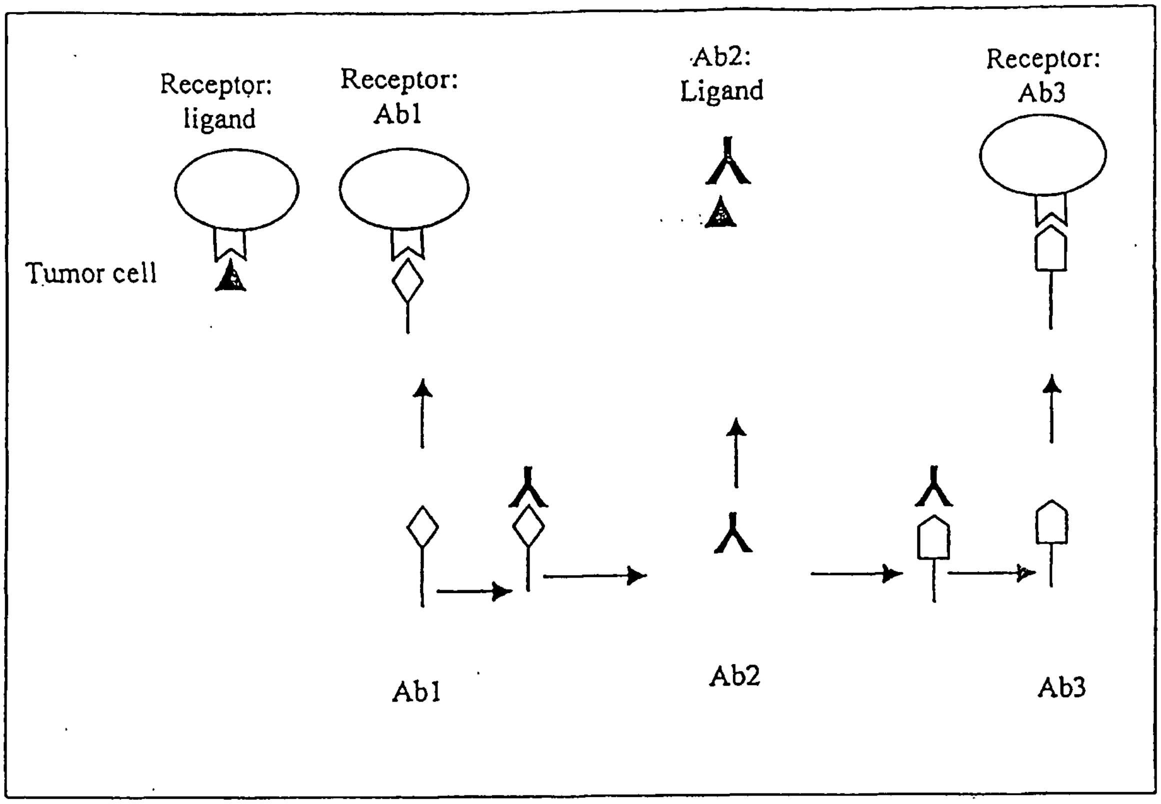

Receptor:
ligand
Receptor:
Ab1
Ab2:
Ligand
Receptor:
Ab3
Tumor cell
Ab1
Ab2
Ab3

Figure 2

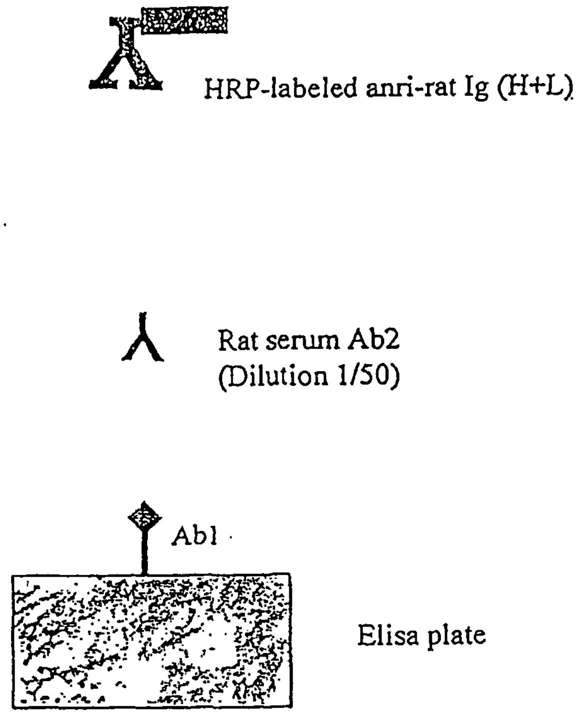


Figure 3

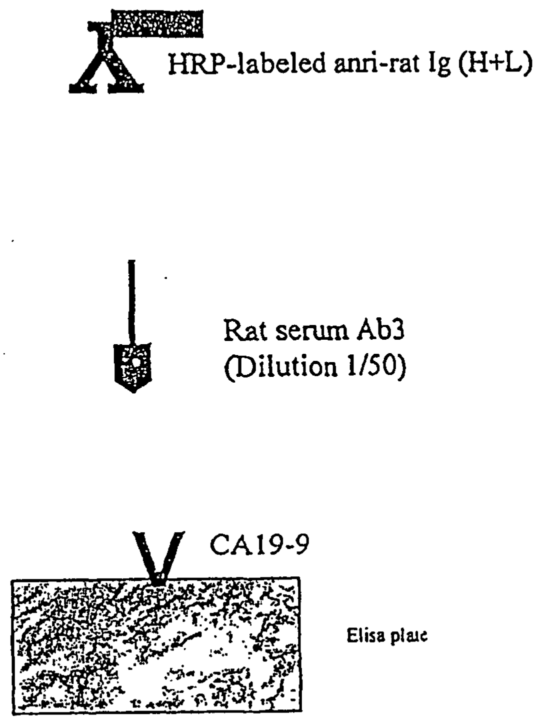


Figure 4

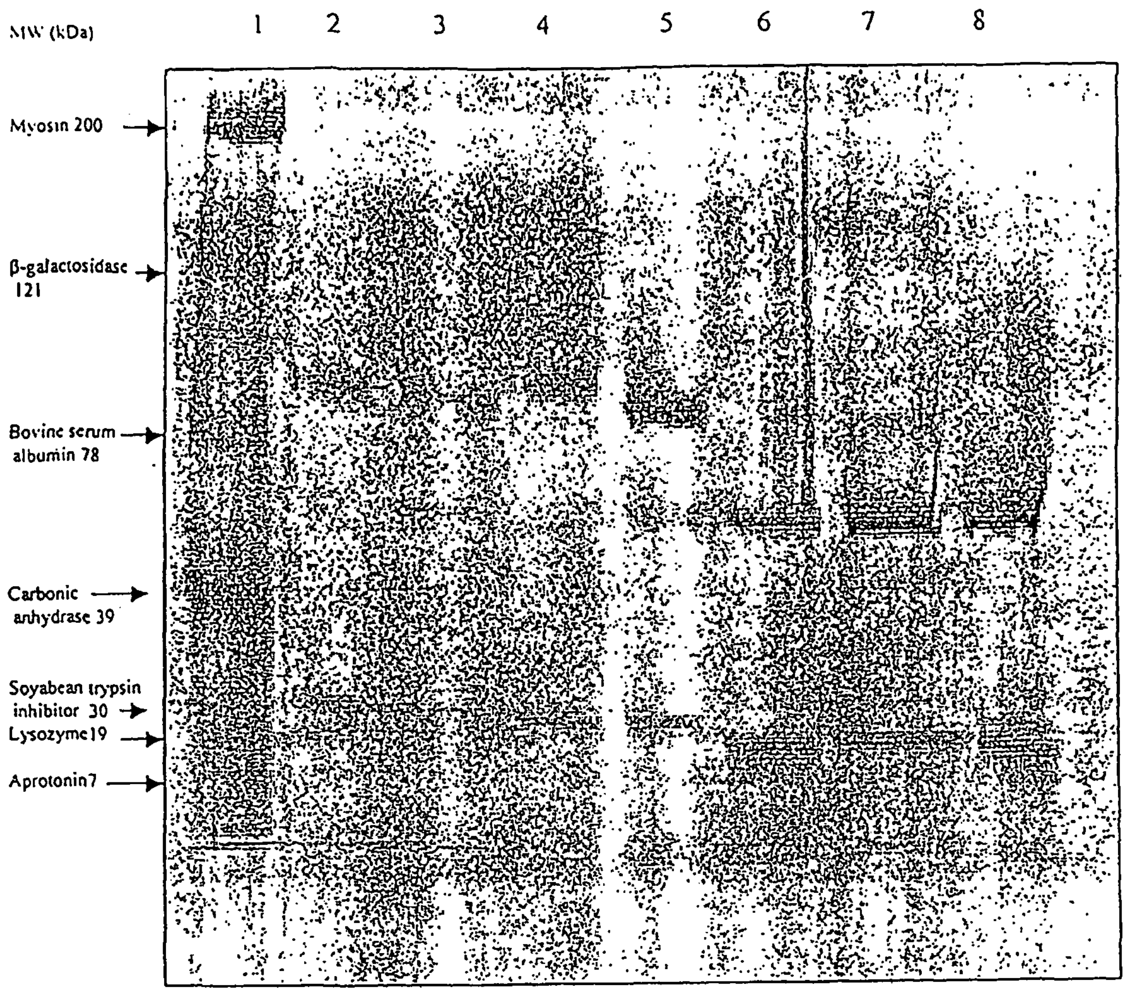

MW (kDa)
1
2
3
4
5
6
7
8
Myosin 200
β-galactosidase 121
Bovine serum albumin 78
Carbonic anhydrase 39
Soyabean trypsin inhibitor 30
Lysozyme19
Aprotonin7

Figure 5

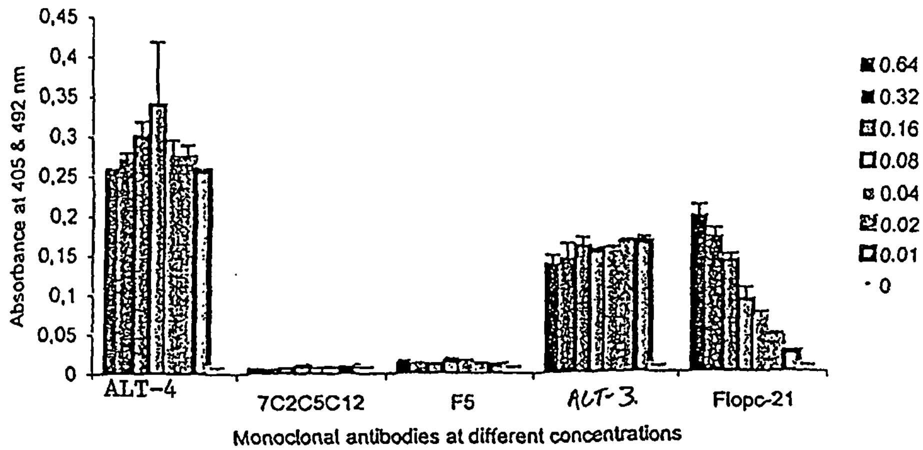

0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Absorbance at 405 & 492 nm
ALT-4
7C2C5C12
F5
ALT-3
Flopc-21
Monoclonal antibodies at different concentrations
0.64
0.32
0.16
0.08
0.04
0.02
0.01
0

Figure 6

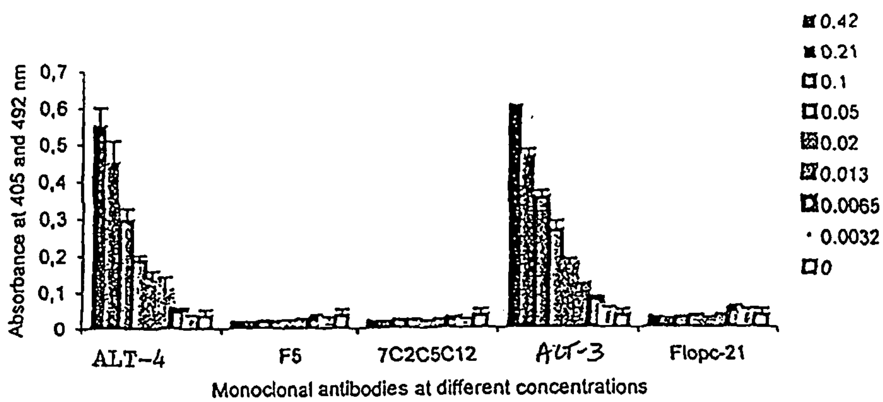

Absorbance at 405 and 492 nm
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ALT-4
F5
7C2C5C12
ALT-3
Flopc-21
Monoclonal antibodies at different concentrations
0.42
0.21
0.1
0.05
0.02
0.013
0.0065
0.0032
0

Figure 7

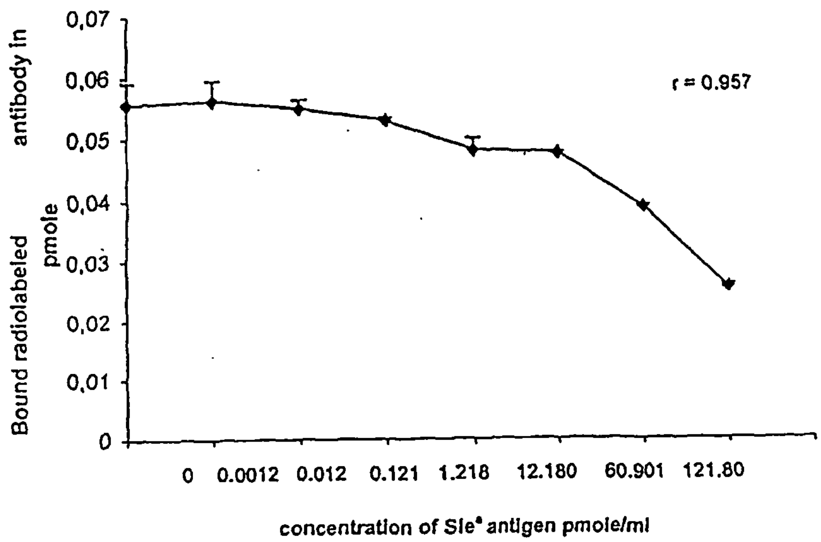

Bound radiolabeled antibody in pmole
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
r = 0.957
0
0.0012
0.012
0.121
1.218
12.180
60.901
121.80
concentration of Sle^a antigen pmole/ml

Figure 8

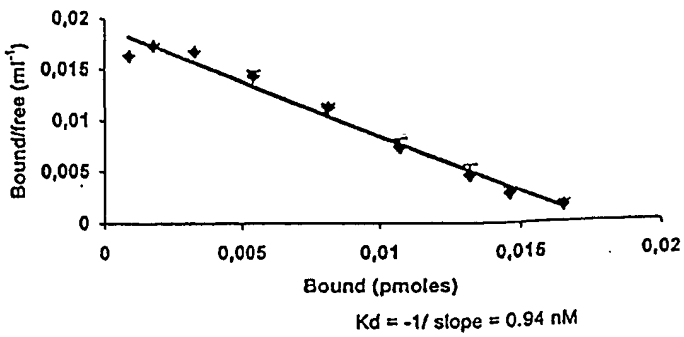
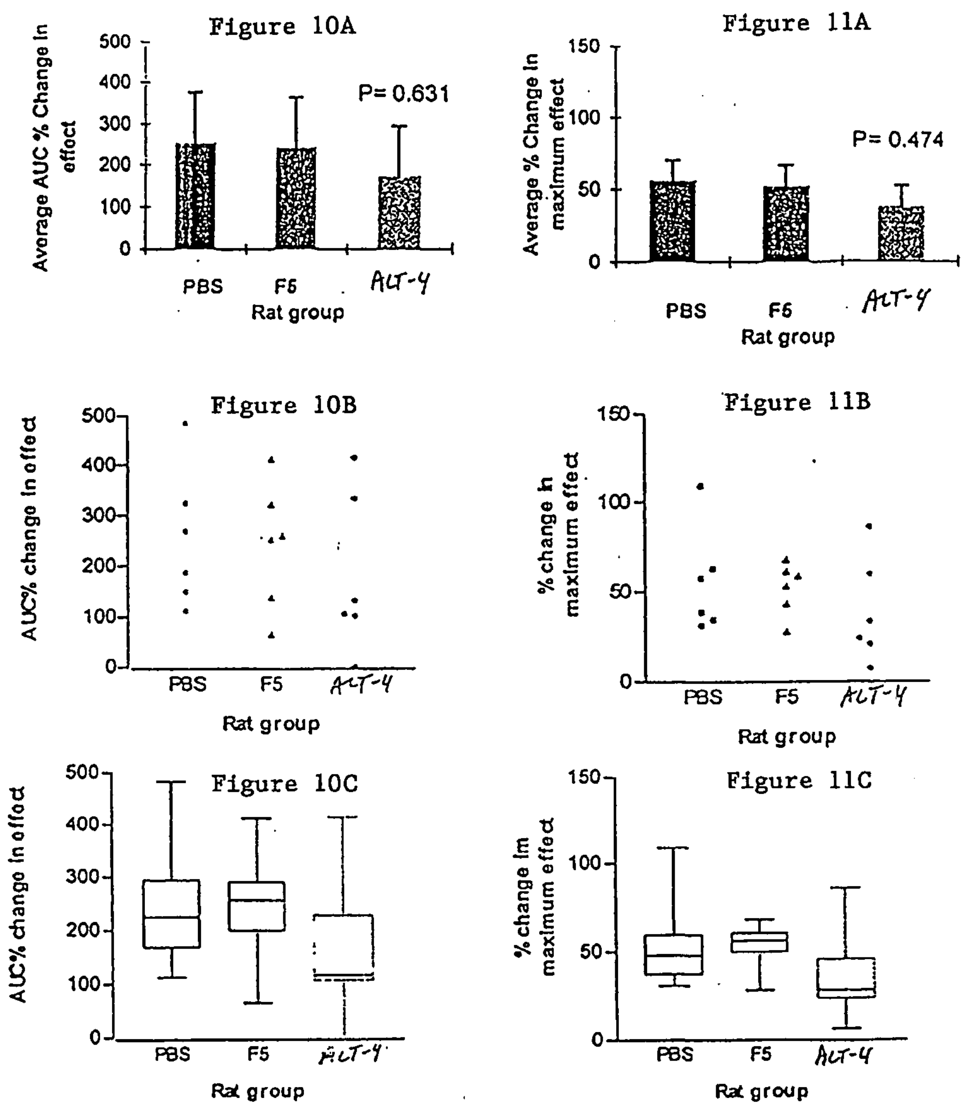
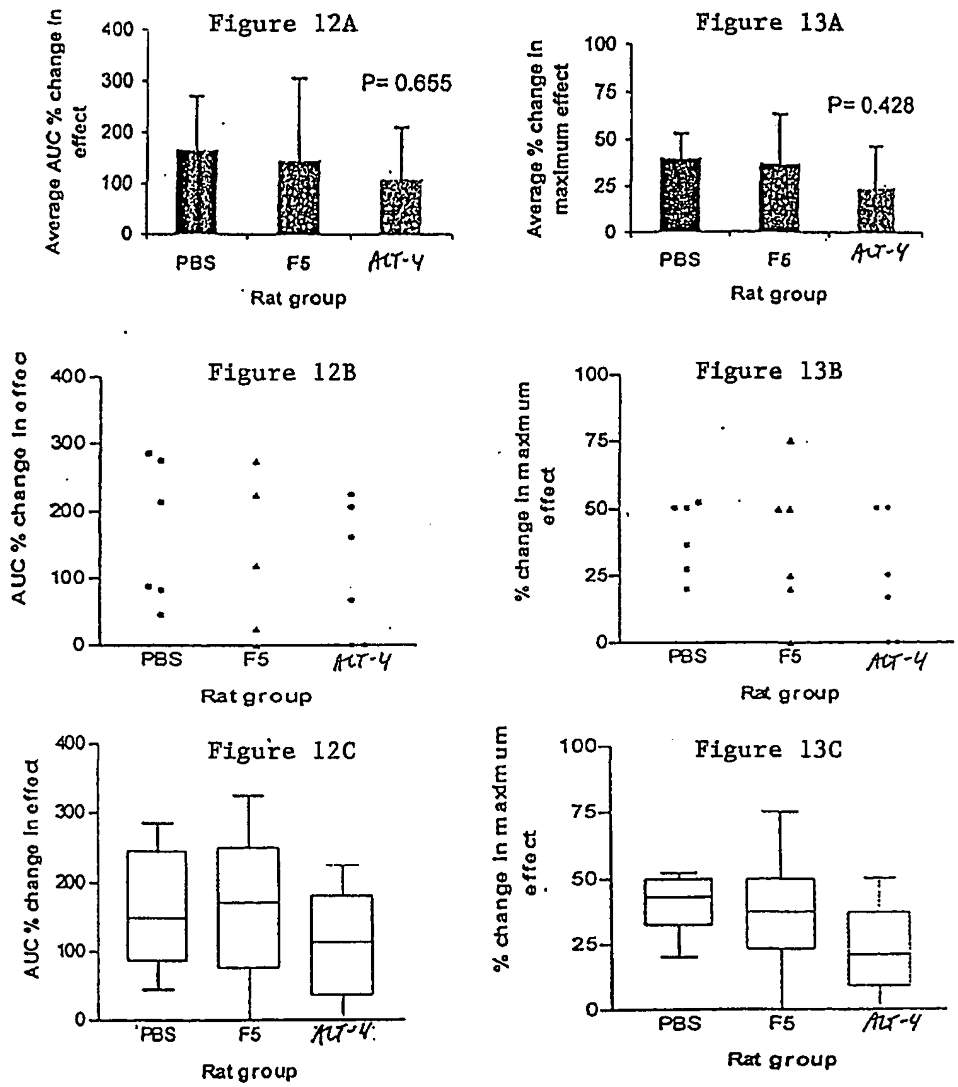

0,02
0,015
0,01
0,005
0
Bound/free (ml⁻¹)
0
0,005
0,01
0,015
0,02
Bound (pmoles)
Kd = -1/ slope = 0.94 nM

Figure 9



Figures 10 and 11



Figures 12 and 13

Figure 14

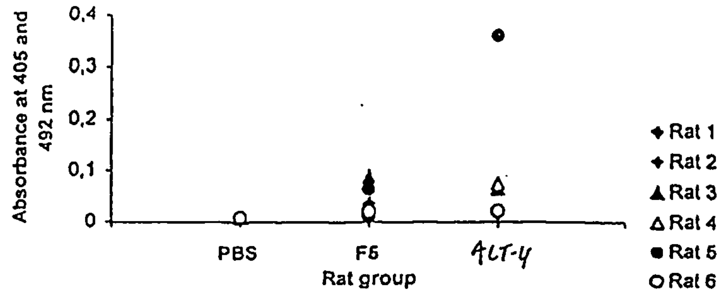


Figure 10B

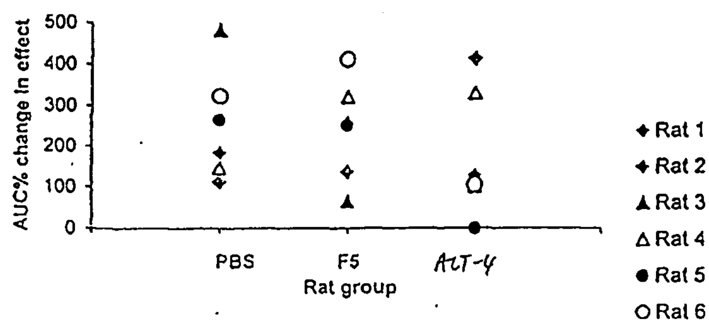


Figure 12B

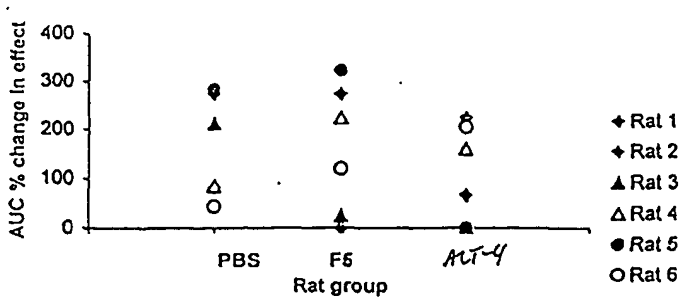
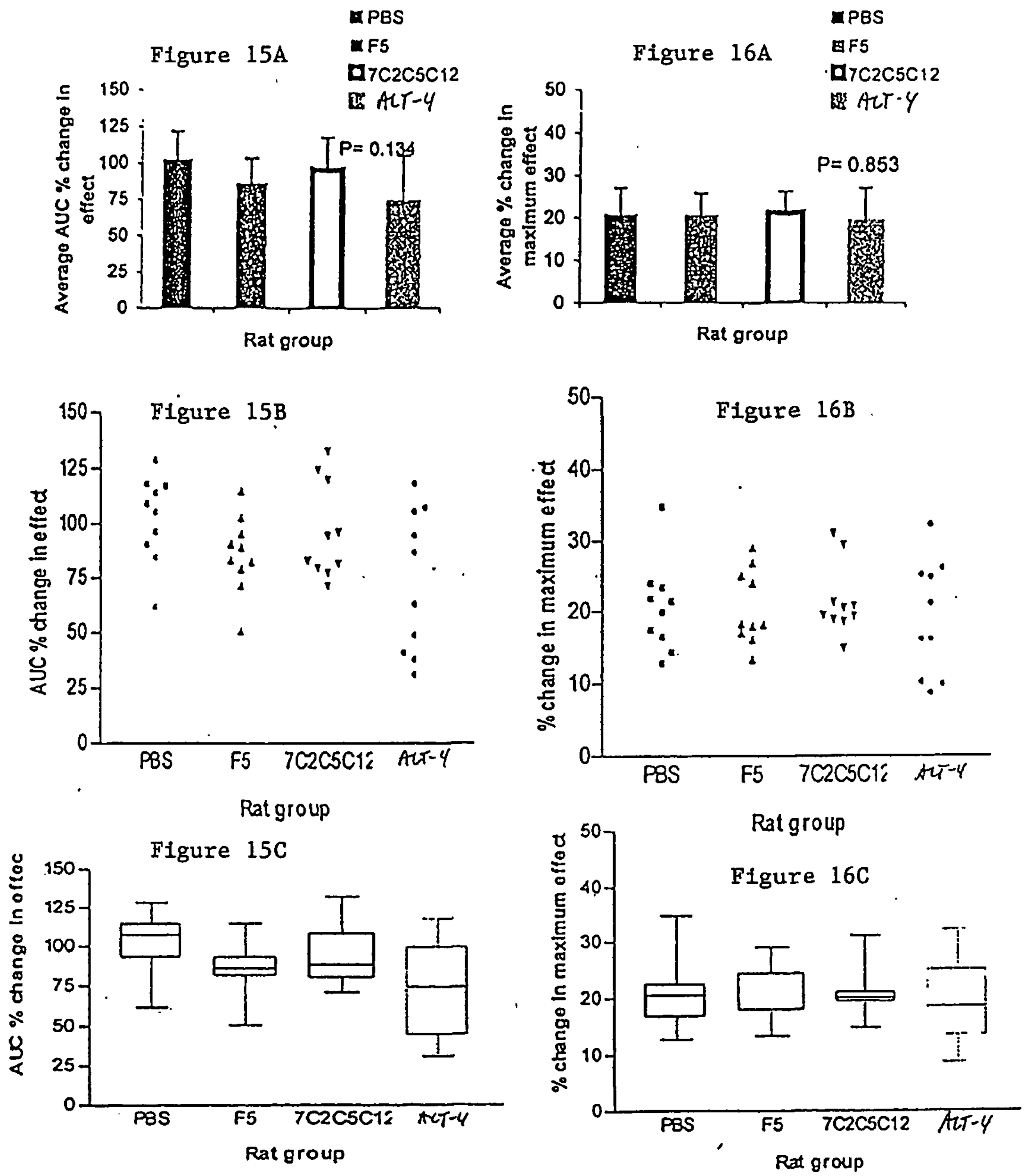
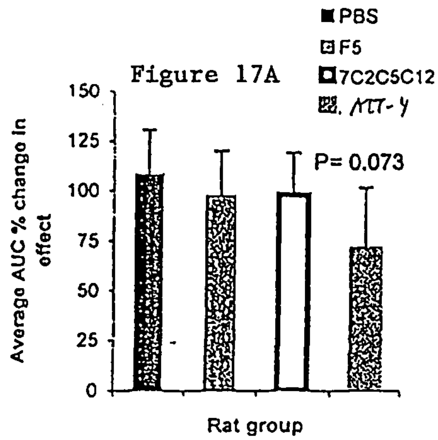
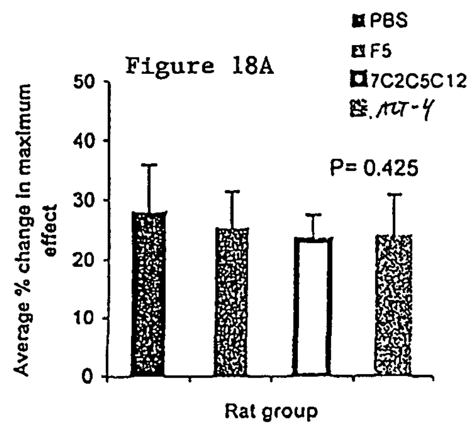


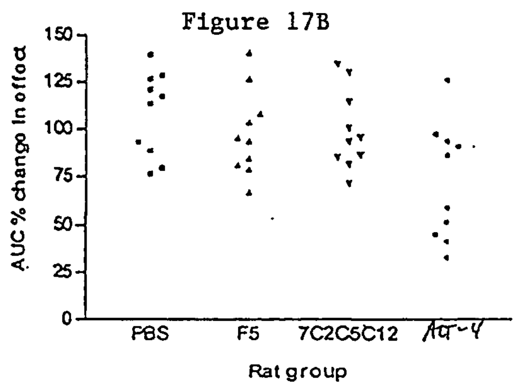
Figure 14

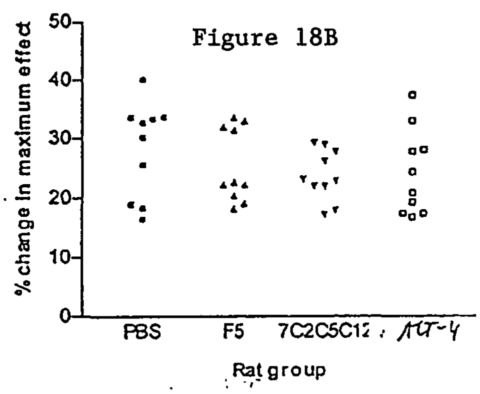


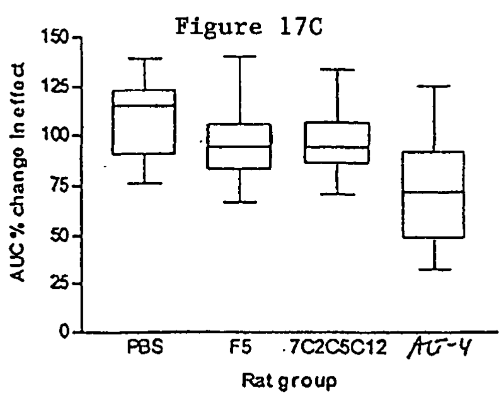
Figures 15 and 16

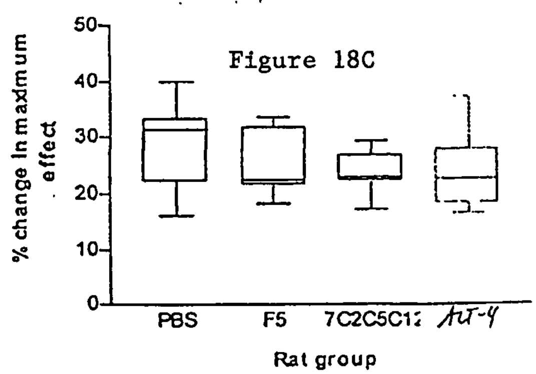












Figures 17 and 18

Figure 19

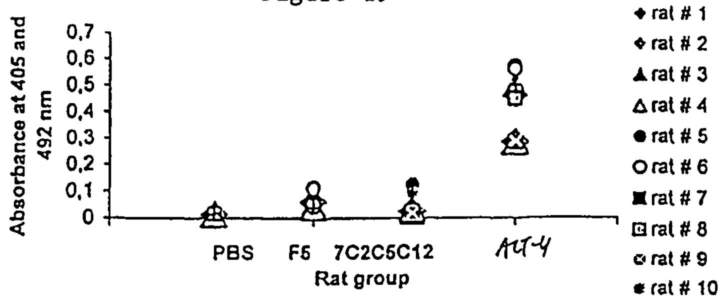


Figure 15B

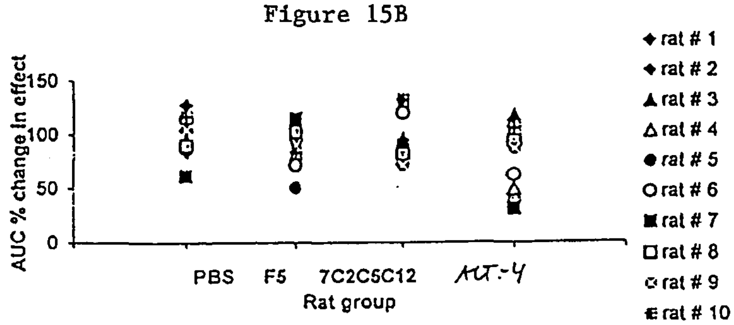


Figure 17B

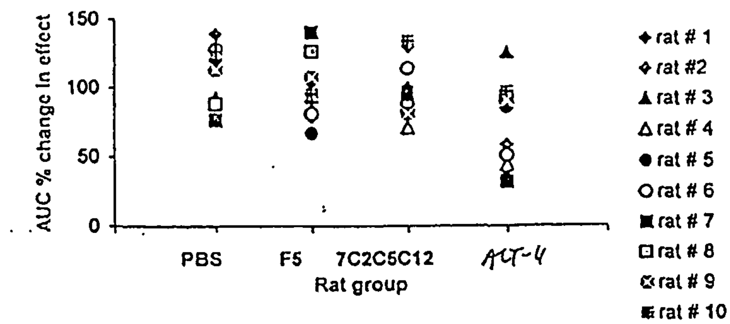


Figure 19

Figure 20A

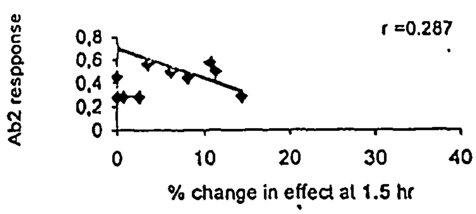


Figure 20B

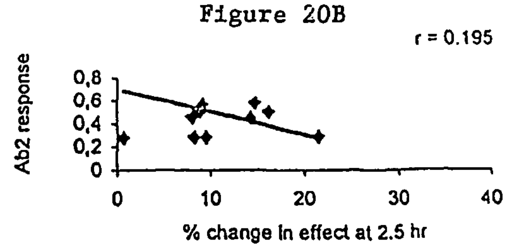


Figure 20C

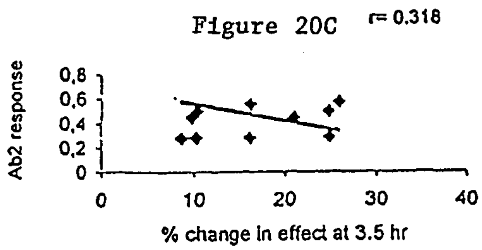


Figure 20D

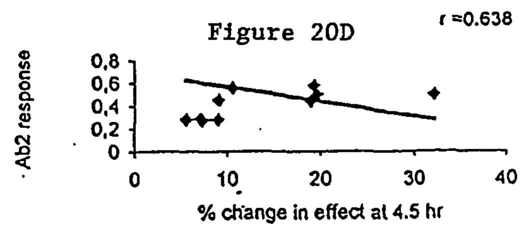


Figure 20E

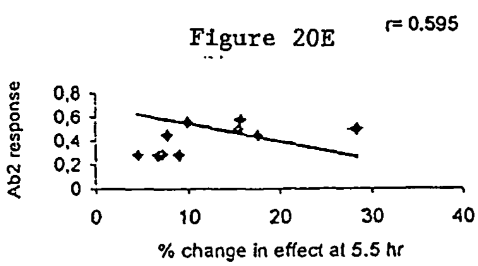


Figure 20F

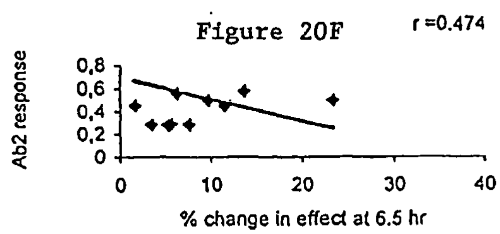


Figure 20G

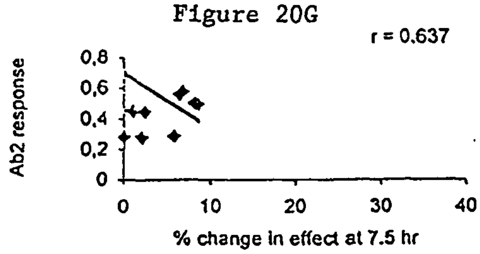


Figure 20H

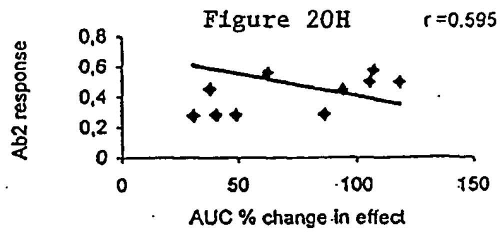
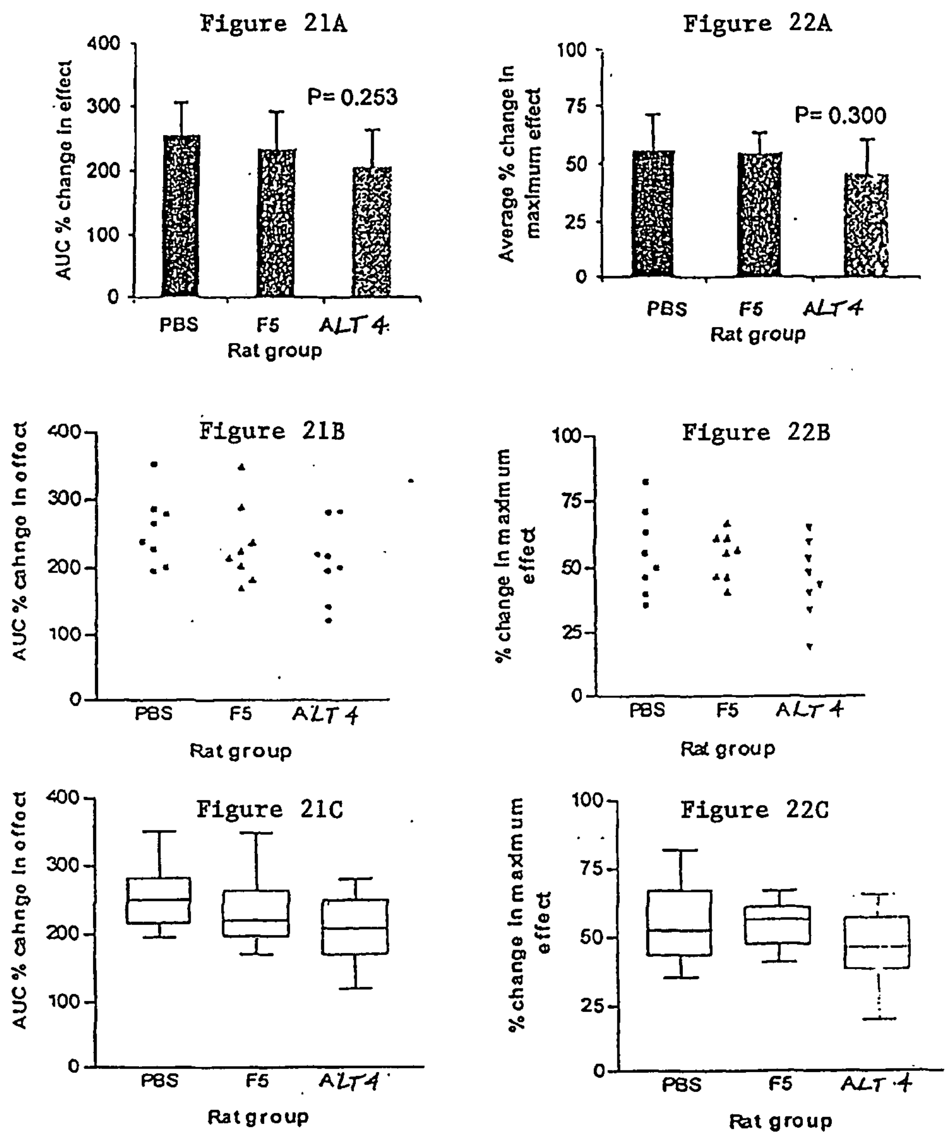
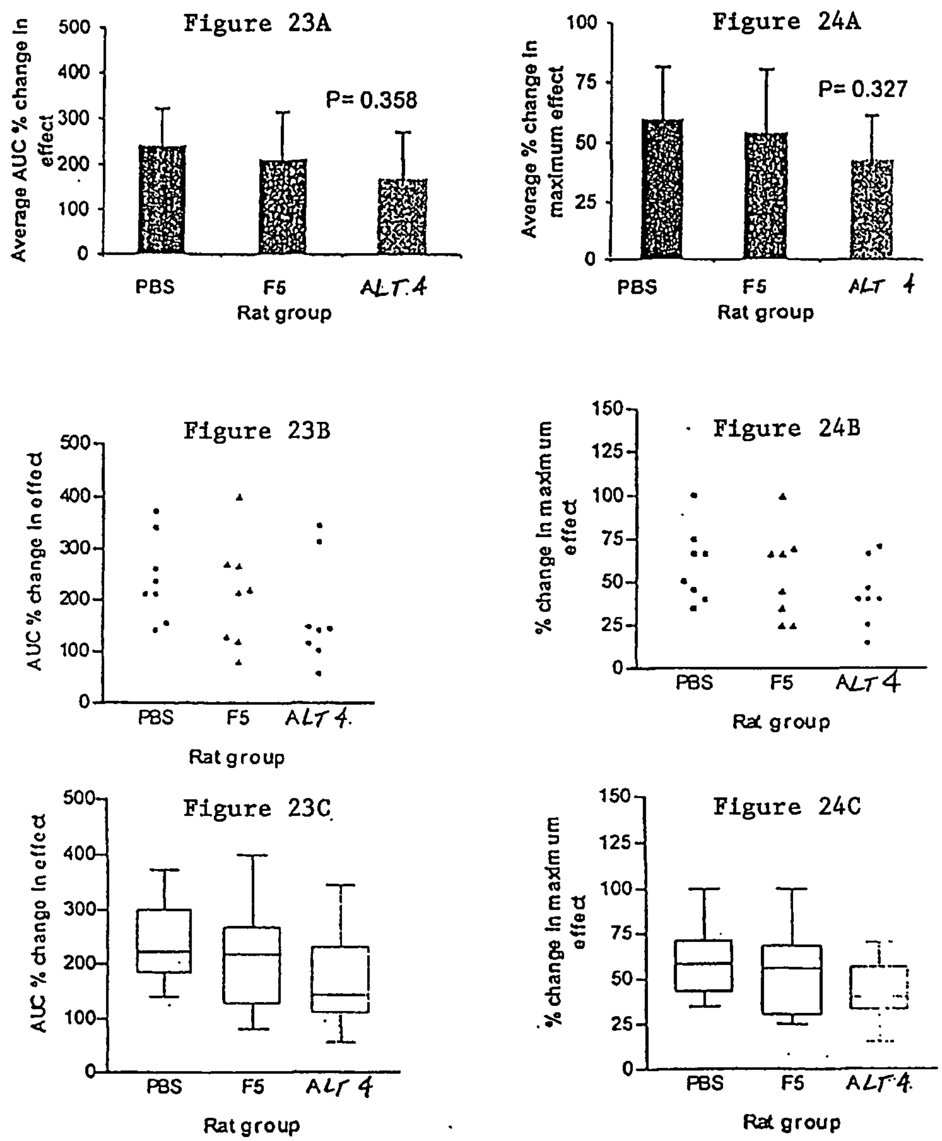


Figure 20



Figures 21 and 22



Figures 23 and 24

Figure 25

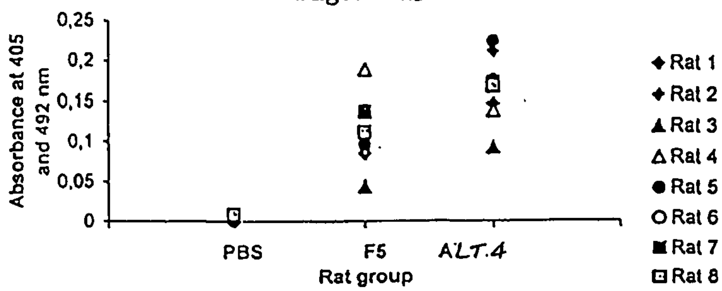


Figure 21B

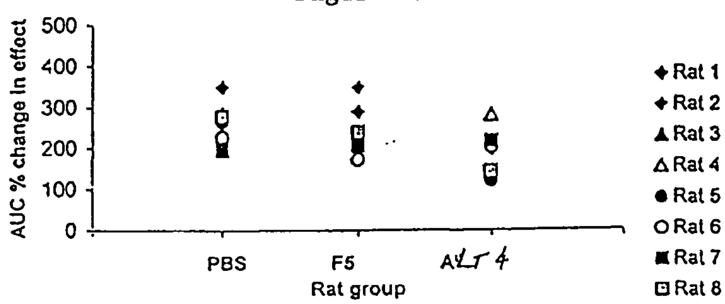


Figure 23B

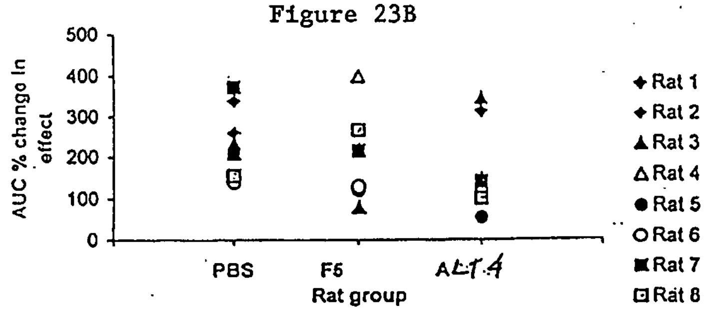


Figure 25

Figure 26A

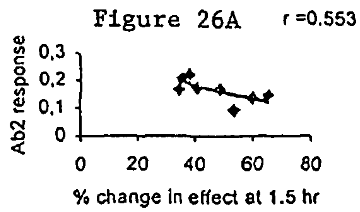


Figure 26B

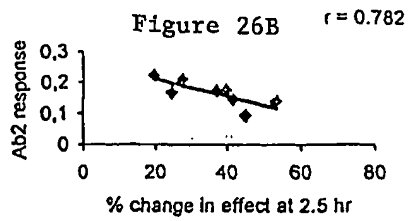


Figure 26C

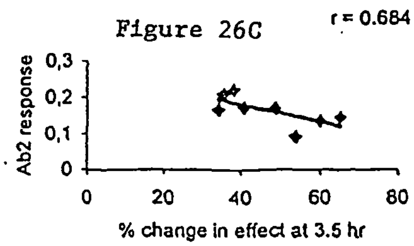


Figure 26D

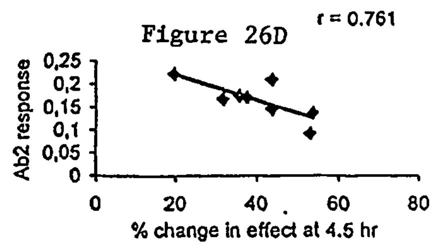


Figure 26E

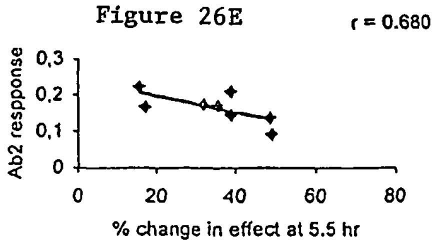


Figure 26F

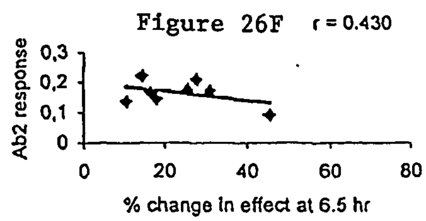


Figure 26G

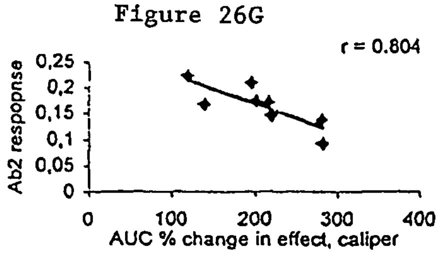


Figure 26

Figure 27A

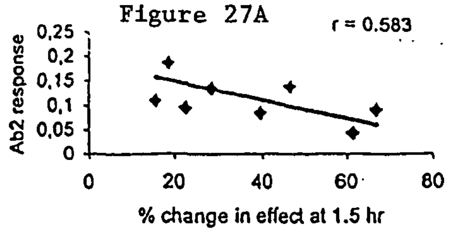


Figure 27B

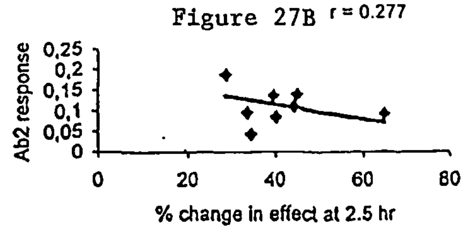


Figure 27C

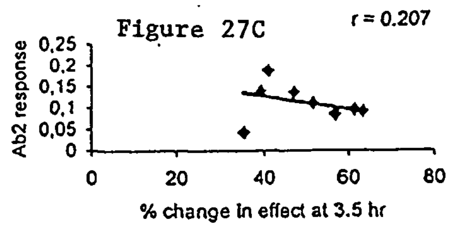


Figure 27D

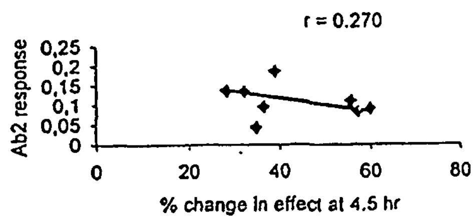
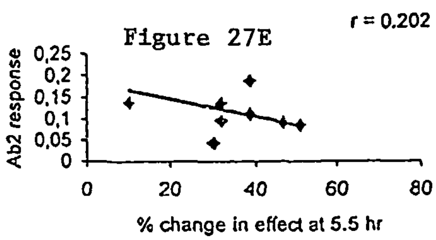


Figure 27E



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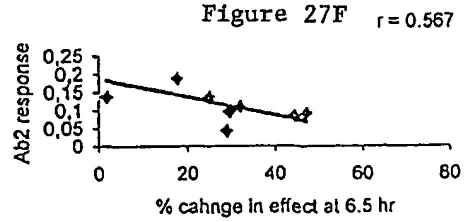


Figure 27G

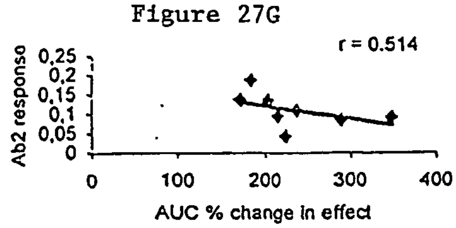
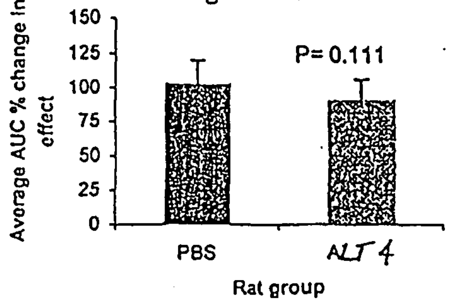


Figure 27

Figure 28A



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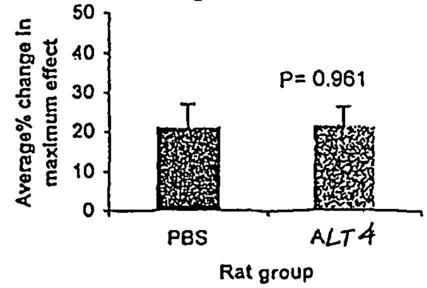

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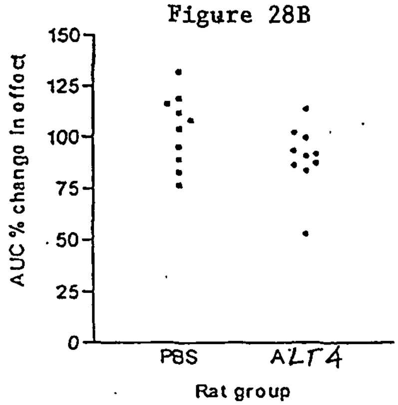

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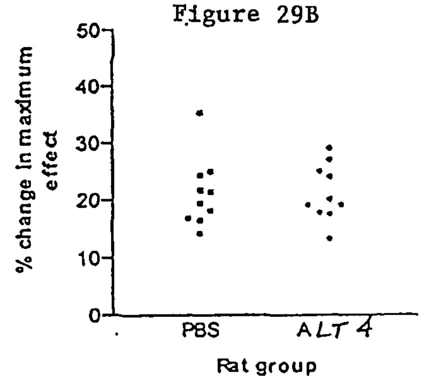
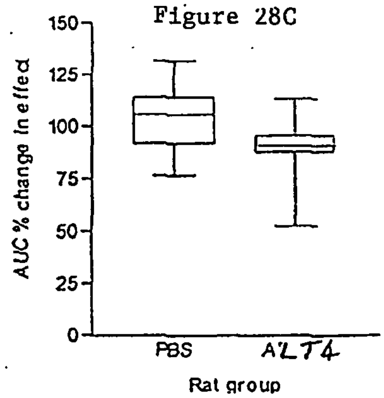


Figure 28C



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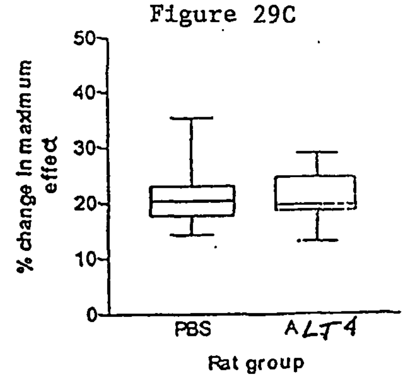


Figures 28 and 29

Figure 30A

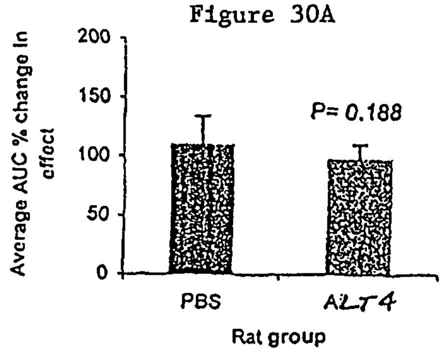


Figure 31A

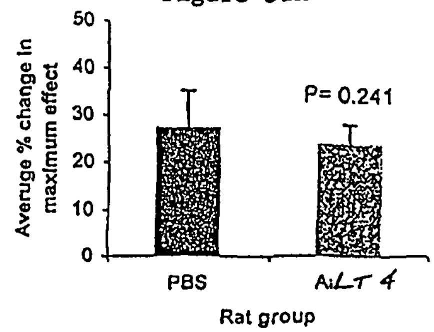


Figure 30B

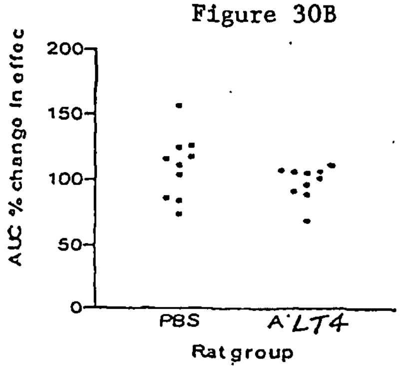


Figure 31B

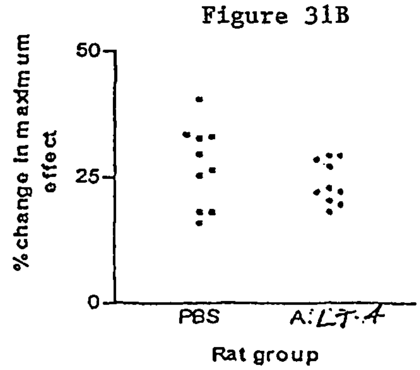
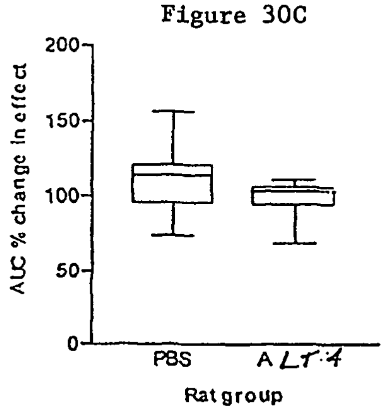


Figure 30C



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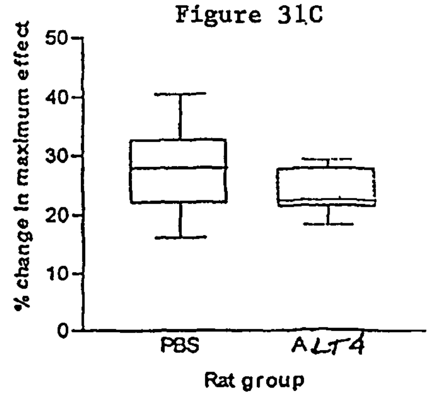


Figures 30 and 31

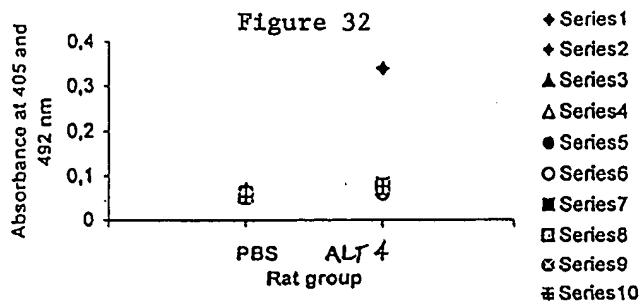


Figure 32
Absorbance at 405 and 492 nm
0,4
0,3
0,2
0,1
0
PBS
ALT 4
Rat group
Series1
Series2
Series3
Series4
Series5
Series6
Series7
Series8
Series9
Series10

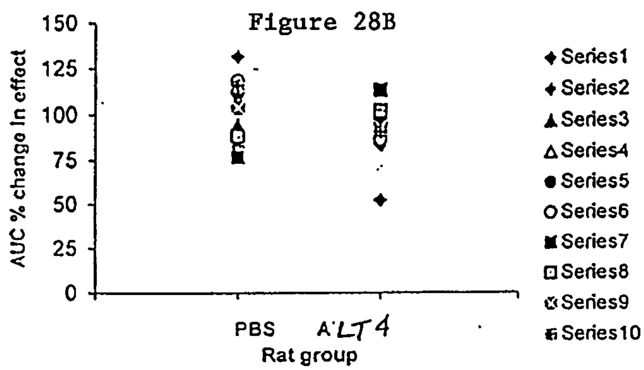


Figure 28B
AUC % change in effect
150
125
100
75
50
25
0
PBS
ALT 4
Rat group
Series1
Series2
Series3
Series4
Series5
Series6
Series7
Series8
Series9
Series10

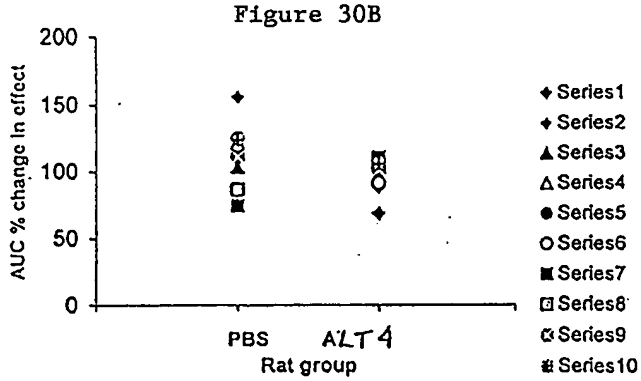
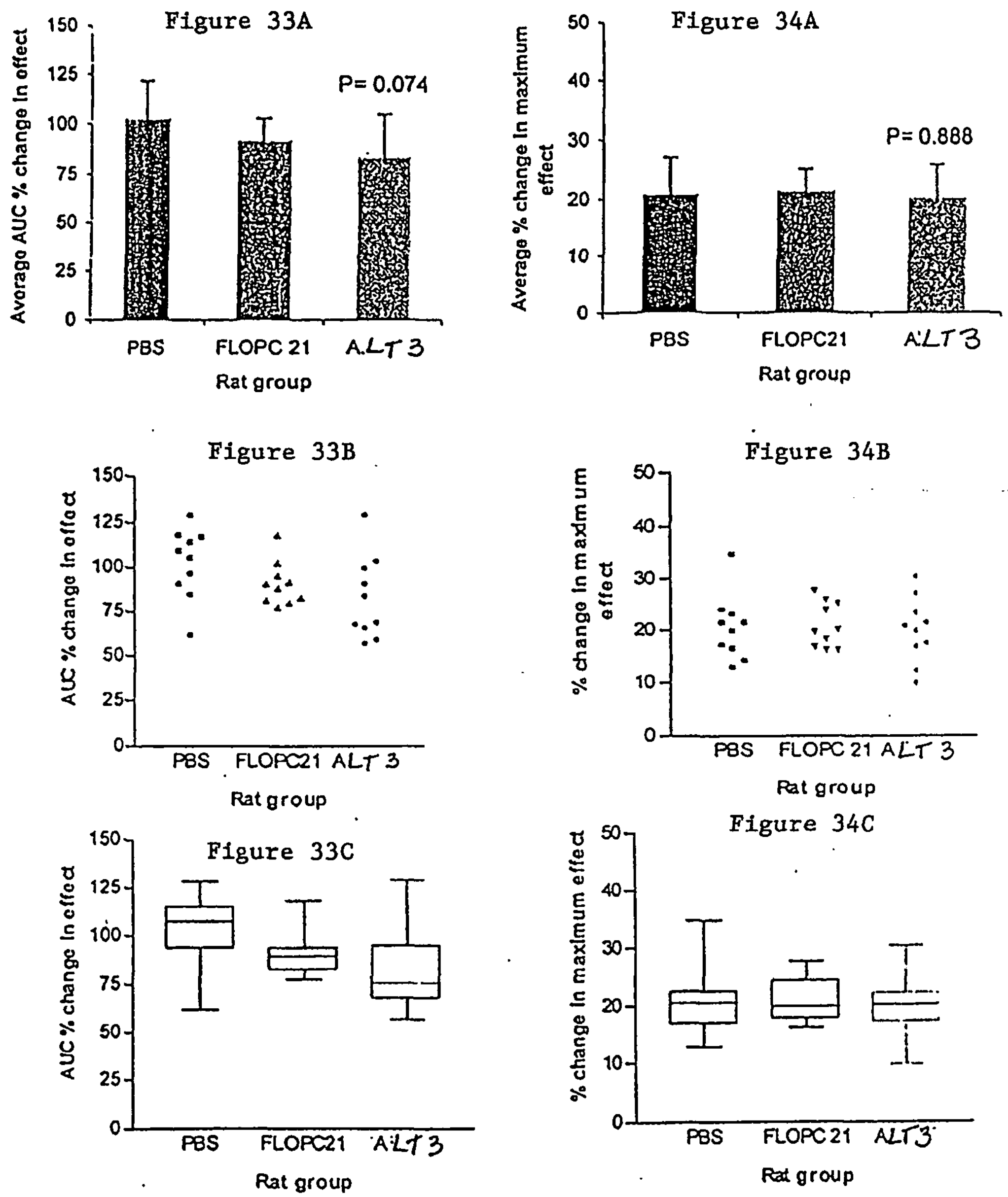


Figure 30B
AUC % change in effect
200
150
100
50
0
PBS
ALT 4
Rat group
Series1
Series2
Series3
Series4
Series5
Series6
Series7
Series8
Series9
Series10

Figure 32



Figures 33 and 34

Figure 35A

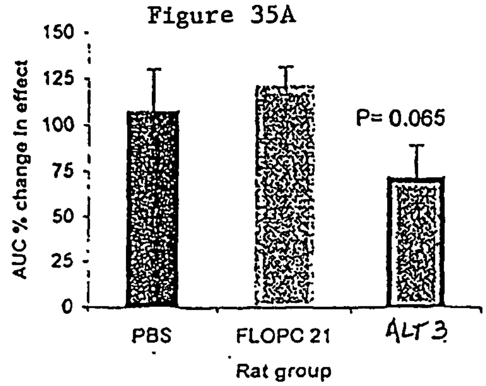


Figure 36A

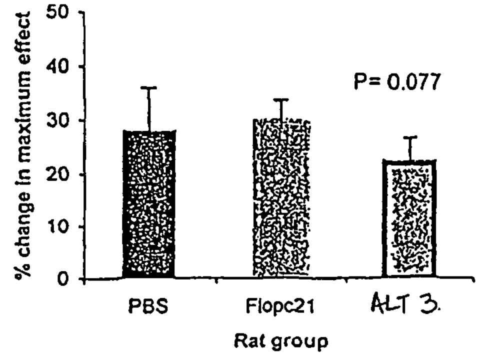


Figure 35B

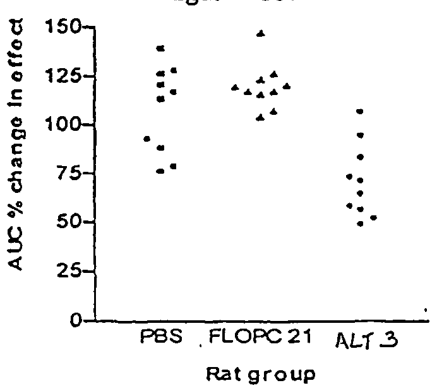


Figure 36B

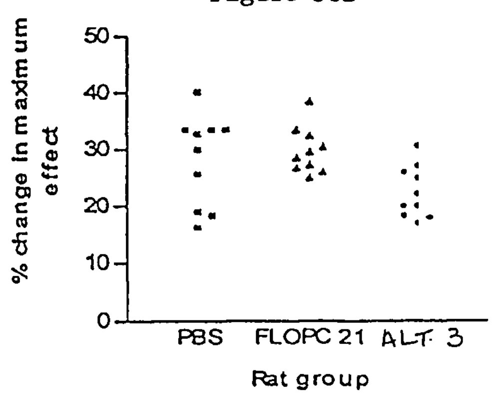


Figure 35C

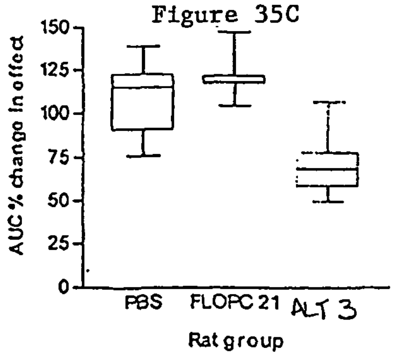
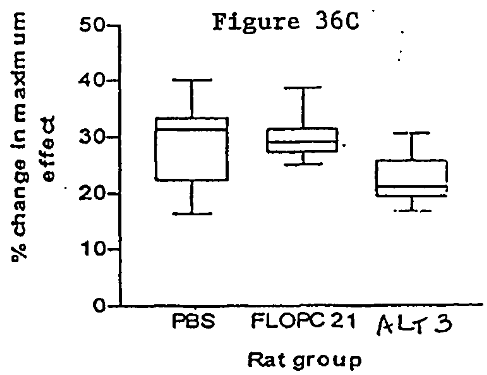


Figure 36C



Figures 35 and 36

Figure 37

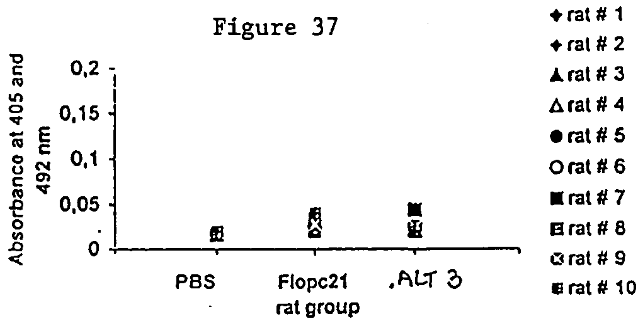


Figure 33B

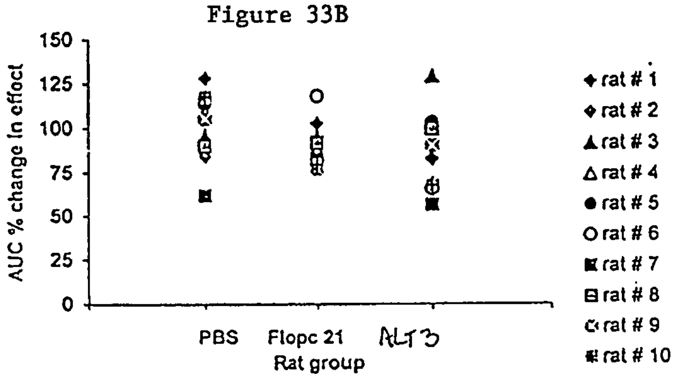


Figure 35B

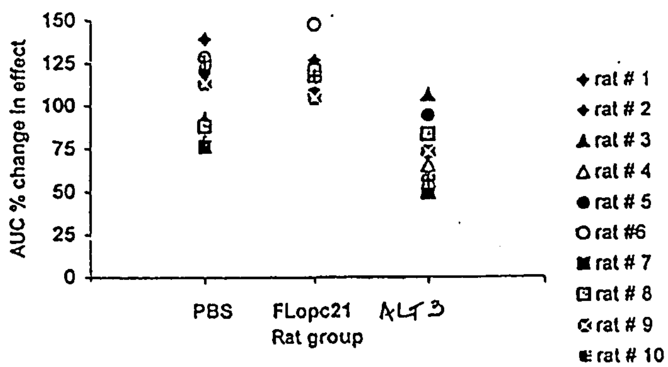


Figure 37

Figure 38A

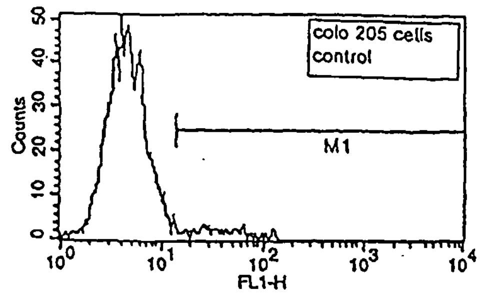


Figure 38B

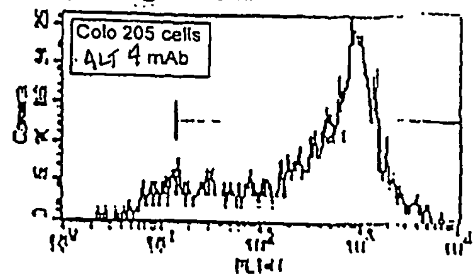


Figure 38C

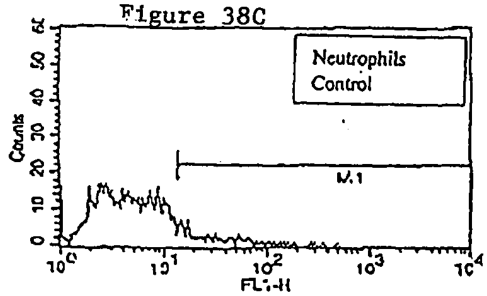


Figure 38D

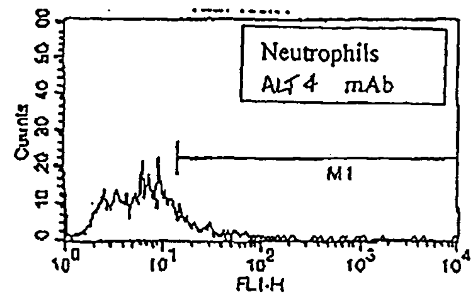


Figure 38E

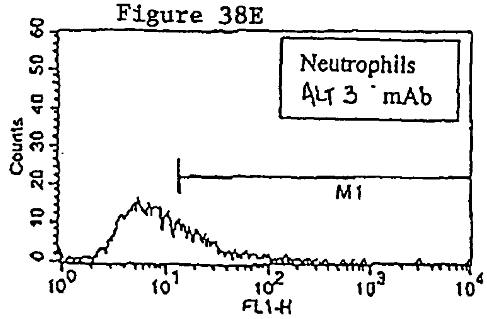


Figure 38F

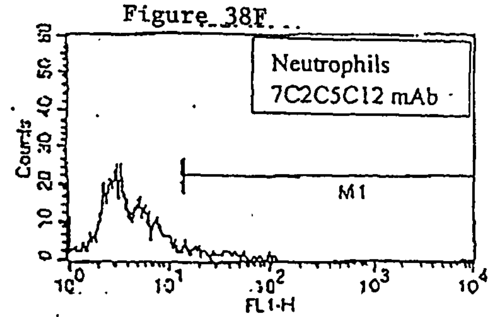


Figure 38G

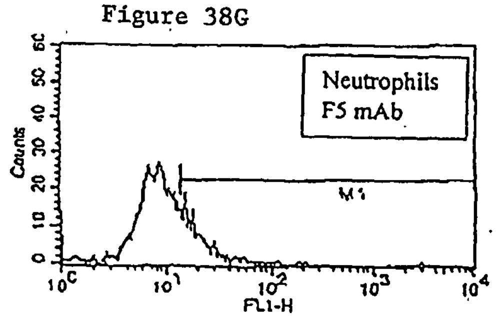


Figure 38H

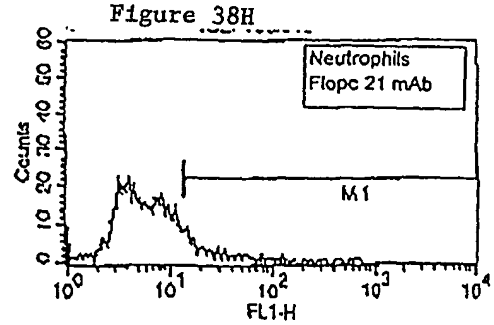


Figure 38

Figure 39A

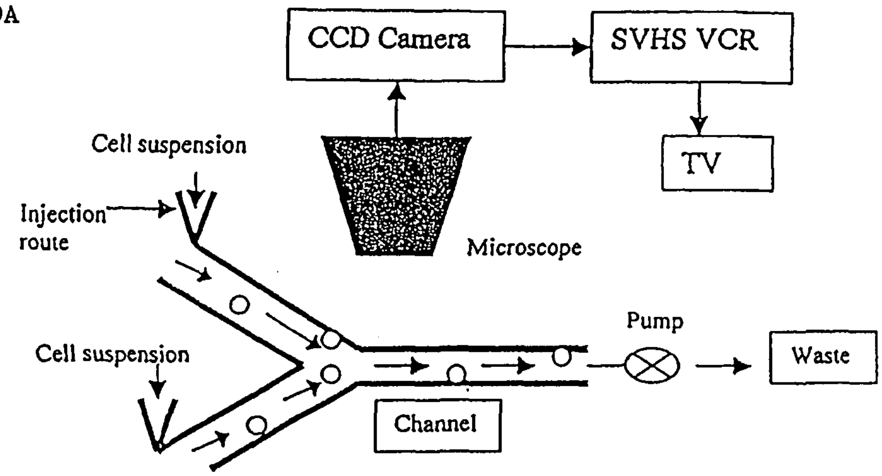


Figure 39B

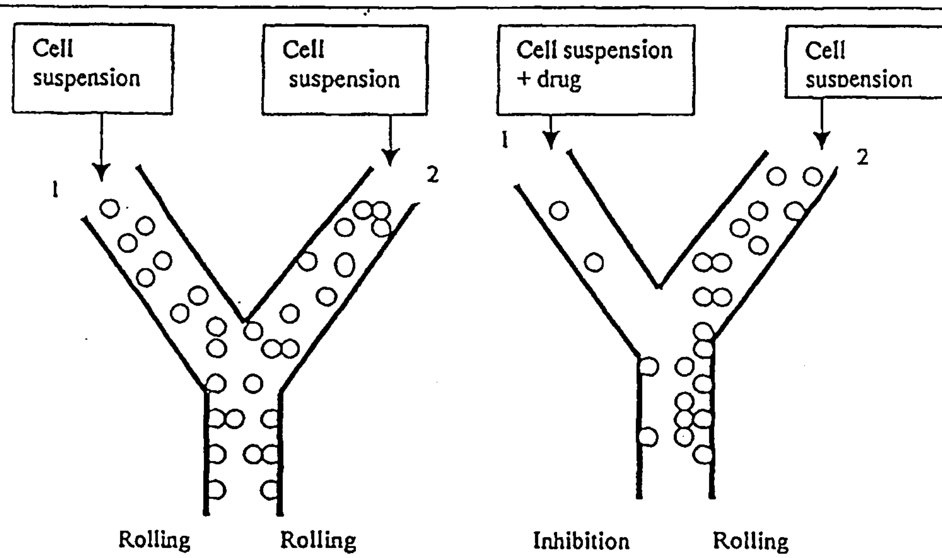


Figure 39

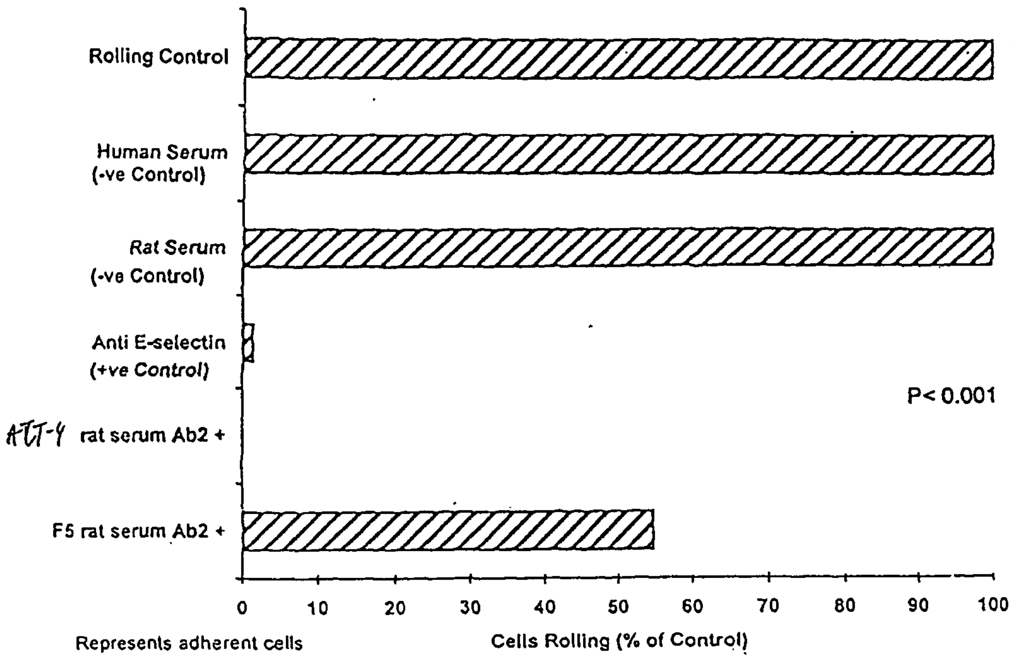

Rolling Control
Human Serum
(-ve Control)
Rat Serum
(-ve Control)
Anti E-selectin
(+ve Control)
P< 0.001
ATT-4 rat serum Ab2 +
F5 rat serum Ab2 +
0
10
20
30
40
50
60
70
80
90
100
Represents adherent cells
Cells Rolling (% of Control)

Figure 40

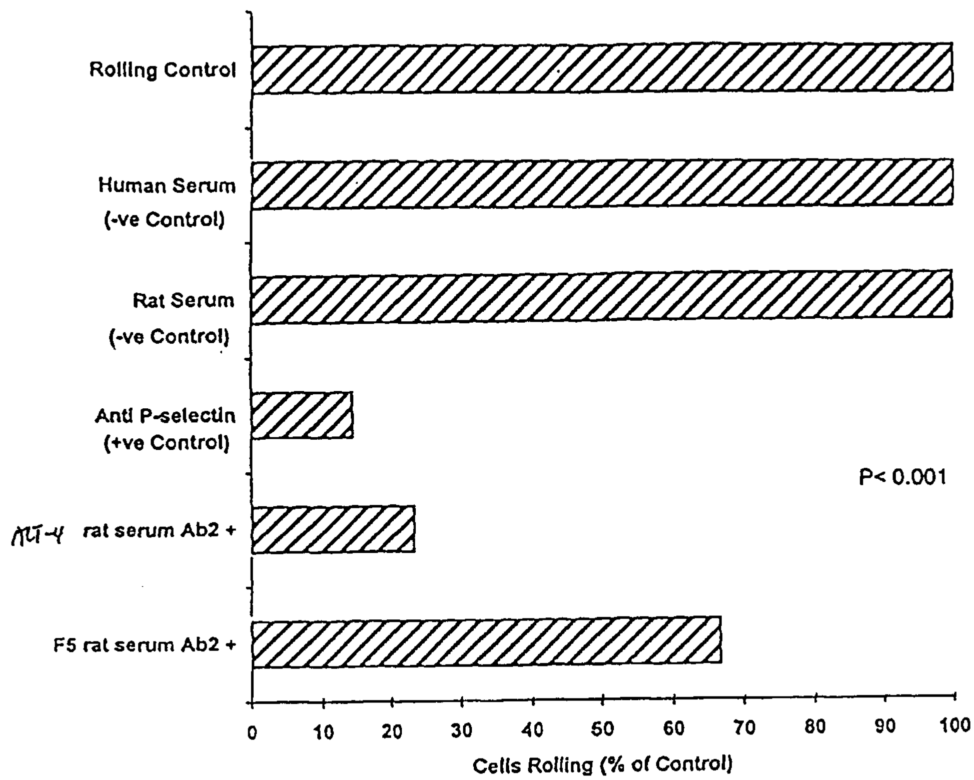

Rolling Control
Human Serum
(-ve Control)
Rat Serum
(-ve Control)
Anti P-selectin
(+ve Control)
rat serum Ab2 +
F5 rat serum Ab2 +
P< 0.001
0
10
20
30
40
50
60
70
80
90
100
Cells Rolling (% of Control)

Figure 41

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/091792 A3

(54) Title: ANTI-ADHESION MOLECULE ANTIBODY TO TREAT INFLAMMATION AND CANCER BY INDUCING THE IDIOTYPIC NETWORK

(57) Abstract: Disclosed are compositions for reducing an immune condition, such as inflammation or cancer, in an animal suffering from the immune condition or predisposed to suffer from the immune condition. The compositions comprise a binding agent that specifically binds to an adhesion molecules expressed on the surface of or released by a blood-borne cell or an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Additional disclosed compositions comprise an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount. Also disclosed are methods for administering such compositions to an animal suffering from or predisposed to suffer from the immune condition.

INTERNATIONAL SEARCH REPORT

Int al Application No
PCT/IB 00/02077

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61P35/00 A61P29/00 //C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRAUGOTT UTE: "L-selectin monoclonal antibody treatment of chronic experimental allergic encephalomyelitis triggers immunomodulation by the idiotypic network." NEUROLOGY, vol. 46, no. 2 SUPPL., 1996, page A296 XP008011512 48th Annual Meeting of the American Academy of Neurology;San Francisco, California, USA; March 23-30, 1996 ISSN: 0028-3878 abstract --- -/--	1-4,6-8, 12-18, 22-25, 27-29, 33-39, 43-47

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2002	13/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	COVONE-VAN HEES, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 56416 A (HERKEL JOHANNES ;COHEN IRUN R (IL); EREZ ALON NETA (IL); ROTTER VA) 17 December 1998 (1998-12-17) page 8, line 26 -page 9, line 5 page 10, line 3-16 ---	1-43
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1997 WALTER ULRIKE M ET AL: "The role of E- and P-selectin in neutrophil and monocyte migration in adjuvant-induced arthritis in the rat." Database accession no. PREV199799611651 XP002225405 abstract	44-47
Y	& EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 27, no. 6, 1997, pages 1498-1505, ISSN: 0014-2980 ---	1-8, 12-29, 33-43
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1996 STEINBACH FRANK ET AL: "The influence of cytokines on the adhesion of renal cancer cells to endothelium." Database accession no. PREV199698681903 XP002225406 abstract	44-47
Y	& JOURNAL OF UROLOGY, vol. 155, no. 2, 1996, pages 743-748, ISSN: 0022-5347 ---	1-5, 9-26, 30-43
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1996 ALLEN MICHAEL H ET AL: "E-selectin binds to squamous cell carcinoma and keratinocyte cell lines." Database accession no. PREV199698778164 XP002225407 abstract	44-47
Y	& JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 106, no. 4, 1996, pages 611-615, ISSN: 0022-202X ---	1-5, 9-26, 30-43
X	QI W ET AL: "Induction of idiotype network to anti-MUC-1 antibody in breast cancer" CHEMICAL ABSTRACTS + INDEXES, AMERICAN CHEMICAL SOCIETY. COLUMBUS, US, vol. 39, March 1998 (1998-03), page 367 XP002155186 ISSN: 0009-2258 abstract ---	1,2, 9-18,22, 23, 30-39, 43-47

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 25647 A (PROCTER & GAMBLE) 23 December 1993 (1993-12-23) page 2, paragraph 3 page 3, paragraphs 1-3	44,46
A	BAUM R P ET AL: "ACTIVATING ANTI-IDIOTYPIC HUMAN ANTI-MOUSE ANTIBODIES FOR IMMUNOTHERAPY OF OVARIAN CARCINOMA" CANCER, AMERICAN CANCER SOCIETY, PHILADELPHIA, PA, US, vol. 73, no. 3, SUPPL, 1 February 1994 (1994-02-01), pages 1121-1125, XP008009941 ISSN: 0008-543X see Conclusions abstract	1-47

International application No.
PCT/IB 00/02077

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-21,46,47 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1,2,14-23,35-47 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1,2,14-23,35-47 (partially)

Present claims 1,14,22,35,44,46 relate to methods and compositions defined by reference to a desirable characteristic or property, namely methods and compositions comprising a (binding) agent (that specifically binds to an adhesion molecule). The claims cover all methods and compositions having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such methods and compositions. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the methods and compositions by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the use of an anti-adhesion molecule antibody (as disclosed in the examples and in claims 12,13) and compositions comprising said antibody (as disclosed in claims 33,34).

Furthermore following claims relate to methods and compositions defined by reference to the following expressions:
a) claims 1,22,46 refer to an immune condition
b) claims 1,15,22,36,45,47 refer to an adhesion molecule
c) claim 1, 22 refer to blood-borne cells
The use of these terms in the present context is considered to lack any technical feature. Moreover said definition could lead to an extremely large number of possible methods and compositions. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the methods and compositions claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely:
a) those parts relating to the methods and compositions to reduce inflammation and cancer as disclosed in the examples and in claims 6,9,27,30
b) those part relating to Sialyl Lewis X, Sialyl Lewis A and selectins as adhesion molecules as disclosed in the examples and in claims 3-5,24-26
c) those parts relating to blood-borne cells as specified in claims 7,8,10,11,28,29,31,132.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int al Application No
PCT/IB 00/02077

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9856416	A	17-12-1998	AU	7672398	A	30-12-1998
			EP	0989861	A1	05-04-2000
			WO	9856416	A1	17-12-1998
WO 9325647	A	23-12-1993	AT	152762	T	15-05-1997
			AU	4398793	A	04-01-1994
			CN	1083096	A ,B	02-03-1994
			DE	69310513	D1	12-06-1997
			DE	69310513	T2	18-12-1997
			DK	646164	T3	03-11-1997
			EP	0646164	A1	05-04-1995
			ES	2103477	T3	16-09-1997
			JP	7508544	T	21-09-1995
			MX	9303584	A1	29-04-1994
			PH	29909	A	16-09-1996
			US	5288431	A	22-02-1994
			WO	9325647	A1	23-12-1993

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CORRECTED VERSION

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(25) Filing Language: English

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60/150,652 25 August 1999 (25.08.1999) US

(71) Applicant: **ALTAREX CORP.** [CA/CA]; 1123 Dentistry Pharmacy Building, University of Alberta, Edmonton, Alberta T6G 2N8 (CA).

(72) Inventors: **MADIYALAKAN, Ragupathy**; 9741-89 Avenue, Edmonton, Alberta T6E 2S1 (CA). **NOUJAIM, Antoine, A.**; 58 Wilkin Road, Edmonton, Alberta T6M 2K4 (CA). **LEVEUGLE, Beatrice**; 9741-89 Avenue, Edmonton, Alberta T6E 2S1 (CA).

(74) Agent: **COTE, France**; Swabey Ogilvy Renault, 1981 McGill College Ave. - Suite 1600, Montréal, Québec H3A 2Y3 (CA).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(48) Date of publication of this corrected version:
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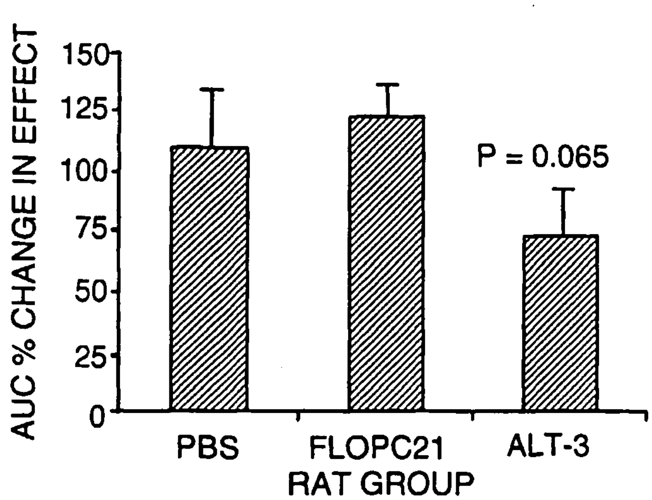
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: THERAPEUTIC IMMUNE CONDITION COMPOSITIONS AND METHODS



(57) **Abstract:** Disclosed are compositions for reducing an immune condition, such as inflammation or cancer, in an animal suffering from the immune condition or predisposed to suffer from the immune condition. The compositions comprise a binding agent that specifically binds to an adhesion molecules expressed on the surface of or released by a blood-borne cell or an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Additional disclosed compositions comprise an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount. Also disclosed are methods for administering such compositions to an animal suffering from or predisposed to suffer from the immune condition.

WO 01/091792 A2

THERAPEUTIC IMMUNE CONDITION COMPOSITIONS AND METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit from U.S. provisional application serial no. 60/150,652 filed August 25, 1999, the entire contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to compositions for the treatment and/or prevention of immune conditions, particularly inflammation and cancer, and methods for making and using such compositions.

Summary of the Related Art

Two immune conditions that affect the health of humans and domesticated animals are inflammation and cancer. Gallin *et al.* (Inflammation: Basic Principles and Clinical Correlates, Raven Press, New York, 1988) teaches that inflammation is a complex protective physiologic response elicited by various stimuli such as infectious agent, localized tissue injury, or other trauma. The inflammatory response involves numerous mediators and various immune cells. Issekutz *et al.* (*Immunology* **88**: 569-576, 1996) teaches that the migration of white blood cells (*i.e.*, leukocytes) from the blood stream through the endothelial cell wall to sites of injury, infection, or immunological reaction is recognized as a characteristic feature and a critical step of the inflammatory response. The accumulation of leukocytes into inflamed tissues occurs as a consequence of endothelial cell activation and leukocyte migration to the inflammatory foci.

Díaz-González and Sánchez-Madrid (*Immunology Today* **19**(4): 1-3, 1998) teach that the process of leukocyte migration requires an interaction between leukocytes and the endothelial cells lining the blood vessel. Leukocyte-endothelial cell interaction is a multi-step process, which involves a cascade of sequential cellular adhesive events that can be divided into four successive steps as illustrated in Figure 1. The initial contact between

leukocytes and the blood vessel wall is a reversible process that involves rolling of leukocytes on the activated endothelium (step one). This rolling of the leukocytes on endothelial cells is facilitated by the adhesion of a selectin molecule (*e.g.*, L-selectin) on the leukocyte to its ligand (*e.g.*, sialyl Lewis X or Sialyl Lewis A) on the endothelial cell. Rolling of leukocytes is followed by activation of the leukocytes by chemokines and cytokines (step two), and firm adhesion of activated leukocytes to the endothelium (step three). Crockett-Torabi and Fantone (*Immunol. Res.* **14**: 237-251, 1995) teach that in the last step, leukocytes extravasate into the surrounding tissues by squeezing themselves between endothelial cell junctions and moving toward the inflammatory foci.

The majority of inflammatory disorders are frequently chronic in duration, and of particular importance are the autoimmune diseases. Autoimmune disease is a group of chronic diseases, in which the immune system not only fail to perform its regular function in protecting the host from invading agents, but also reacts particularly against some of the host autoantigens. This autoimmune response is initiated by cellular, humoral, or both mechanisms, and leads to disease with local inflammation and tissue damage. Multiple sclerosis (MS), systemic lupus erythematosus (SLE), Crohns disease, and rheumatoid arthritis (RA) are just few examples of autoimmune diseases.

Pitzalis *et al.* (*Annals of Rheumatic Diseases* **53**: 287-288, 1994) discuss the role of adhesion molecules in the pathogenesis of RA and other autoimmune diseases. Leukocyte infiltration into tissues is believed to be a major component of the pathologic process leading to joint injury in chronic arthritis, including rheumatoid arthritis. Therefore, adhesion molecules represent another attractive immunotherapeutic target for intervention in RA and other autoimmune diseases.

Selectins and their carbohydrate ligands have been considered very important targets in inflammatory conditions because of their important role in the initial contact between leukocytes and the vascular endothelium at sites of inflammation. McMurray, R.W. (*Seminars in Arthritis & Rheumatism* **25**: 215-234, 1996) teaches that selectins are carbohydrate binding adhesion molecules that share a common structural component consisting of a $Ca^{(2+)}$ dependent N-terminal lectin binding domain. McEver, R.P. (*Curr Opin Immunol* **6**: 75-84, 1994) teaches that selectins recognize and bind to specific carbohydrate structures on ligands that are composed of sialic acid, fucose, galactose, mannose, and/or an anionic sulfate or phosphate ester moieties such as sialyl Lewis X (SLe(x)), sialyl Lewis A (SLe(a)), and related structures.

Munro *et al.* (*American Journal of Pathology* **141**(6): 1397-1405, 1992) teach that the carbohydrate ligand, SLe(x)) [NeuAca2-3Galb1-4(Fuca1-3) GlcNAc-] is expressed on the cell surface of monocytes, a polymorphonuclear leukocyte (PMNL), and on approximately 10% or peripheral blood T lymphocytes. SLe(x) is recognized by all selectin adhesion molecules. The SLe(x) isomeric epitope, SLe(a) [NeuAca2-3Galb1-3(Fuca1-4) GlcNAc-] (Zhang *et al.*, *Tumor Biology* **18**: 175-187, 1997), is recognized and bound to by E and P-selectins, and possibly L-selectin (Paavonen and Renkonen, *American Journal of Pathology* **141**(6): 1259-1261, 1992). Takada *et al.* (*BiochemBiophys Res Commun* **179**: 713-19, 1991) teach that SLe(a) is absent on most peripheral blood cells.

Blocking selectins or their ligands with antibodies or oligosaccharides has been attempted in various animal models of inflammation. For example, Weiser *et al.* (*Shock* **5**(6): 402-407, 1996) describe that in rats that had undergone hour of hindlimb tourniquet ischemia, the administration of anti-P-selectin monoclonal antibody (mAb), PB1.3, allegedly reduced hindlimb injury, but not leukosequestration. Yamada *et al.* (*European Journal of Pharmacology* **346**: 222-224, 1998) describe that the same anti-P-selectin mAb PB1.3 allegedly reduced infarct size by approximately 38% (2 mg/kg) and 28% (5 mg/kg) in rabbits undergoing 30 minutes of ischemia followed by 5 hour reperfusion. Tojo *et al.* (*Glycobiology* **6**(4): 463-9, 1996) allege that the anti-rat-P-selectin mAb, ARP2-4, attenuated infarct size in rat myocardial ischemia and reperfusion injury model. Ohnishi *et al.* (*Immunopharmacology* **34**: 161-170, 1996) describe that the anti-rat-P-selectin mAb, ARP2-4, allegedly reduced the footpad swelling and inhibited the associated polymorphonuclear leukocyte (PMNL) accumulation when administered to rats who had undergone dermal injury induced by Arthus reaction.

Altavilla *et al.* (*European Journal of Pharmacology* **270**: 45-51,1994) allege that the use of the anti-E-selectin mAb, BBIG-E5, resulted in the reduction in infarct size as well as PMNL accumulation in rodent model.

A study on endotoxin-induced uveitis in mice (Whitcup *et al.*, *Clinical Immunology and Immunopathology* **83**(1): 45-52, 1997) reported that neutrophil migration into the eye was allegedly effectively inhibited with a combination of anti- E-selectin and anti-P-selectin monoclonal antibodies administered intraperitoneally, either before or after endotoxin injection. In this study, anti-E-selectin antibody alone allegedly had little effect on endotoxin-induced uveitis, while anti-P-selectin mAb decreased ocular inflammation by 37% when administered at the time of or 6 hours after endotoxin injection.

Ma *et al.* (*Circulation* **88**: 649-658, 1993) allege that a reduction in infarct size as well as PMNL accumulation was observed with the use anti-L-selectin mAb, DREG-200, in a feline model. Pizcueta and Luscinskas (*American Journal of Pathology* **145**(2): 461-467, 1994) disclose an *in vivo* murine model of experimentally induced chronic inflammation of the peritoneum in which anti-L-selectin mAb (MEL-14) was infused intravenously (i.v.) to investigate the role of L-selectin in the recruitment of mononuclear leukocytes to chronic sites of inflammation (48 hours). Results from this study showed an allegedly reduced accumulation of macrophages and lymphocytes by 60% and 90%, respectively, at 48 hours. Similarly, MEL-14 mAb allegedly dramatically inhibited granulocyte influx by 80% at 6 hours and by 50% at 24 and 48 hours.

Most of the studies on selectin-carbohydrate ligands have targeted Sialyl Lewis X (SLe(x))) oligosaccharide molecule. Tojo *et al.* (*Glycobiology* **6**(4): 463-9, 1996) describe the effect of SLe(x)-oligosaccharide on rat myocardial ischemia and reperfusion injury. In this study, the administration of SLe(x)-oligosaccharide allegedly caused significant reduction in infarct size without affecting hemodynamic parameters or circulating leukocyte numbers.

Han *et al.* (*Journal of Immunology* **155**(8): 4011-5, 1995) describe the effect of a soluble SLe(x) on ear edema and necrosis in rabbits where the ear vascular supply was occluded for 6 hours, and allowed to reperfuse later. In this study, tissue edema and necrosis were allegedly significantly reduced in animals treated with SLe(x) (25 mg / kg bolus i.v. followed by 50 mg/kg infusion over 10 hour) immediately upon reperfusion or after a 1-hour delay, but not in animals for whom the treatment was delayed 4 or 12 hours.

Schmid *et al.* (*Journal of Heart & Lung Transplantation* **16**(10): 1054-1061, 1997) evaluated the inhibitory effect of CY-1503, an analogue of SLe(x), on PMNL migration and reperfusion injury in canine left lung allografts (35 mg/kg i.v. bolus) where the recipient contralateral right pulmonary artery and bronchus were ligated. In this study, allograft gas exchange and hemodynamics results allegedly demonstrated that the group treated with CY-1503 had a reduction in PMNL adhesion, migration, and subsequent reperfusion injury in preserved canine lung allografts. Birnbaum *et al.* (*Journal of Molecular & Cellular Cardiology* **29**(8): 2013-25, 1997) discloses that the treatment with CY-1503 (30 mg/kg i.v. bolus) allegedly did not limit infarct size or prevent the "no-reflow" phenomenon in rabbits who were subjected to 30 minutes of coronary artery occlusion and 4 hour of reperfusion. Yet additional studies demonstrated that SLe(x) oligosaccharide allegedly reduced infarct

size by 83% and inhibited PMNL adhesion to endothelial cells in the feline model (Buerke *et al.*, *Journal of Clinical Investigation* **93**: 1140-1148, 1994); and in the canine model it allegedly reduced infarct size by 67% and ameliorated PMNL accumulation in myocardium by 63% (Lefer *et al.*, *Circulation* **90**: 2390-2401, 1994).

In addition, the effect of anti- SLe(x) mAb and the expression of SLe(x) were examined in rat hearts subjected to 30 minutes of ischemia followed by reperfusion (Yoshinori *et al.*, *Journal of Pathology* **180**: 305-310, 1996). Reperfusion of ischaemic myocardial tissue in this study allegedly resulted in enhanced expression of SLe(x) on the luminal surface of vascular endothelial cells, as well as myocytes. Furthermore, the *in vivo* administration of anti- SLe(x) mAb significantly reduced the extent of the myocardial infarction.

The mortality from cancer often arises not from the primary tumor site, but from the secondary tumor sites caused by migration of metastatic cancer cells. Many cancer cells, like many blood cells, express sialyl Lewis X and/or sialyl Lewis A (Ravindranath et al., Cancer **79(9)**:1686-1697, 1997; Tomlinson *et al.*, *Int. J. Oncol.* **16(2)**:347-353, 2000; Skorstengaard *et al.*, *J. Urol.* **161(4)**:1316-1323, 1999; Groves *et al.*, *Am. J. Pathol.* **143(4)**:1220-1225, 1993; Farmer *et al.*, *Head Neck* **20(8)**:726-731, 1998)

Fukuda *et al.*, *Cancer Res.* **60(2)**:450-456, 2000) allege that a peptide mimic of E-selectin ligand inhibits the lung colonization of melanoma cells expressing sialyl Lewis X.

Although numerous studies have attempted to use antibodies against adhesion molecules to reduce immune conditions in animal models, because such high dosages of antibody are required, these approaches may result in antibody-dependent cellular cytotoxicity (ADCC) or antibody-mediated toxicity in human patients. Thus, there remains a need to develop safer approaches to reducing immune conditions that allow for the administration of lower dosages of binding agents, such as antibodies, to adhesion molecules.

SUMMARY OF THE INVENTION

The invention provides compositions for reducing an immune condition. In addition, the invention provides methods of administration of an agent, preferably a binding agent (such as an antibody) to an adhesion molecule, particularly an adhesion molecule involved in the extravasion of white blood cells or cancer cells from the blood into the surrounding tissue, to an animal suffering from the immune condition (or predisposed to develop the immune condition). The relatively low dosage of the agent according to the compositions and methods of the invention allow for a reduction in the risk of a treated animal developing antibody-dependent cellular cytotoxicity (ADCC) or antibody-mediated toxicity.

Accordingly, in a first aspect, the invention provides a method for reducing an immune condition in an animal suffering from the immune condition, comprising administering to the animal a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the animal is a domesticated. More preferably, the animal is a mammal, such as a human.

In some embodiments of the first aspect of the invention, the adhesion molecule is a molecule comprising a carbohydrate epitope. In certain embodiments, the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A. In some embodiments, the adhesion molecule is a selectin. In preferred embodiments, the selectin is P-selectin or E-selectin.

In certain embodiments of the first aspect of the invention, the immune condition is inflammation. In certain embodiments, the blood-borne cell is a white blood cell. In certain embodiments, the white blood cell is selected from the group consisting of a lymphocyte, a neutrophil, a polymorphonuclear leukocyte, and a monocyte.

In certain embodiments of the first aspect of the invention, the immune condition is cancer. In certain embodiments, the blood-borne cell is a cancer cell. In certain embodiments, the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

In various embodiments of the first aspect, the binding agent is an antibody, such as a monoclonal antibody. Preferably, the binding agent is a murine monoclonal antibody.

In certain embodiments of the first aspect of the invention, the immune response results in the generation of an antibody that specifically binds to the binding agent. In certain embodiments, the immune response results in the generation of an antibody that specifically binds to the adhesion molecule. In some embodiments, the immune response comprises a humoral and a cellular immune response.

In various embodiments of the first aspect, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

In a second aspect, the invention provides a composition for reducing an immune condition in an animal suffering from the immune condition comprising a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the animal is a domesticated. More preferably, the animal is a mammal, such as a human.

In some embodiments of the second aspect of the invention, the adhesion molecule is a molecule comprising a carbohydrate epitope. In certain embodiments, the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A. In some embodiments, the adhesion molecule is a selectin. In preferred embodiments, the selectin is P-selectin or E-selectin.

In certain embodiments of the second aspect of the invention, the immune condition is inflammation. In certain embodiments, the blood-borne cell is a white blood cell. In certain embodiments, the white blood cell is selected from the group consisting of a lymphocyte, a neutrophil, a polymorphonuclear leukocyte, and a monocyte.

In certain embodiments of the second aspect of the invention, the immune condition is cancer. In certain embodiments, the blood-borne cell is a cancer cell. In certain embodiments, the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

In various embodiments of the second aspect, the binding agent is an antibody, such as a monoclonal antibody. Preferably, the binding agent is a murine monoclonal antibody.

In certain embodiments of the second aspect of the invention, the immune response results in the generation of an antibody that specifically binds to the binding agent. In certain embodiments, the immune response results in the generation of an antibody that specifically binds to the adhesion molecule. In some embodiments, the immune response comprises a humoral and a cellular immune response.

In various embodiments of the second aspect, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal. In certain embodiments, the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

In a certain preferred embodiment of the second aspect of the invention, the composition also comprises a pharmaceutically-acceptable carrier.

In a third aspect, the invention provides a therapeutic composition comprising an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount. Preferably, the agent is a binding agent.

In a fourth aspect, the invention provides a method for reducing an immune condition in an animal suffering from cancer or inflammation or predisposed to suffer from cancer and inflammation, comprising administering to the animal a therapeutically effective amount of an agent that is capable of reducing inflammation and reducing cancer in an animal Preferably, the agent is a binding agent.

According to the invention, agents of the invention, such as binding agents found to specifically bind to an adhesion molecule, may be administered in a therapeutically effective amount to an animal suffering from inflammation, cancer, or predisposed to suffer from inflammation or cancer. For example, a therapeutically effective amount of an antibody that specifically binds to Sialyl Lewis X may be administered with a pharmaceutically-acceptable carrier (*e.g.*, physiological sterile saline solution) via any route of administration to a patient

suffering from inflammation in an attempt to alleviate any resulting disease symptom (*e.g.*, necrosis). For example, the agent of the invention may be delivered subcutaneously, intravenously, intraperitoneally, intra-arterially, intradermally, or intra-muscularly. Pharmaceutically-acceptable carriers and their formulations are well-known and generally described in, for example, Remington's Pharmaceutical Sciences (18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation illustrating the different steps in the adhesion cascade between leukocytes and endothelial cells (Díaz-González and Sánchez-Madrid, *Immunology Today* **19**(4): 1-3, 1998).

Figure 2 is a schematic representation of the idiotypic cascade, a non–limiting theory of the present invention, showing reactivity with ligand and/or receptor by mirror image. In this schematic, Ab1 (the injected antibody) which specifically binds to and blocks the receptor antigen on the surface of a cell (*e.g.*, a tumor cell) is depicted as having a diamond shape. The injected animal then generates an immune response to Ab1 resulting in the production of Ab2, depicted in this schematic as having a "Y" shape. Ab2 in this schematic specifically binds to the natural ligand (depicted as a solid triangle) of the receptor. The presence of Ab2 results in the production by the immune system of the animal a third antibody, Ab3, which is depicted as having a pentagonal shape that mimicks the natural ligand of the receptor.

Figure 3 is a schematic representation illustrating the use of the enzyme linked immunosorbent assay (ELISA) for the detection of Ab2 antibodies. Note that where the Ab1 antibody is a murine antibody which is injected into a rat, this ELISA will also detect rat anti-mouse antibodies (RAMA).

Figure 4 is a schematic representation illustrating the use of ELISA for the detection of Ab3. Note that the CA19-9 antigen bears the Sialyl Lewis A antigen.

Figure 5 is a schematic representation showing SDS-polyacrylamide Gel Electrophoresis of purified monoclonal antibodies (10% acrylamide Tris-HCl under reducing conditions). Molecular weight (MW) is indicated at the left for the standard loaded into Lane 1. The antibodies are as follows: F5 (IgM) (Lane 2; purified by Mannan binding protein affinity chromatography); 6C2C5C12 (IgM) (Lane 3; purified by the euglobulin precipitation method); ALT-4 (also called HB1; IgM) (Lane 4; purified by Mannan binding protein affinity chromatography); Alt-4 (also called HB1; IgM) (Lane 5; old sample of purified antibody); Flopc-21 (IgG3) (Lane 6; purified by protein A affinity chromatography) ALT-3 (IgG3) (Lane 7; purified by the euglobulin precipitation method); and ALT-3 (IgG3) (Lane 8; old sample of purified antibody).

Figure 6 is a schematic representation showing the binding of various representative, non-limiting murine monoclonal antibodies (7C2C5C12, F5, ALT-3, and Flopc-21 (at the

indicated concentrations) to immobilized tumor antigen CA19-9, a non-limiting representative antigen of the invention.

Figure 7 is a schematic representation showing the binding of various representative, non-limiting murine monoclonal antibodies (7C2C5C12, F5, ALT-3, and Flopc-21 (at the indicated concentrations) to immobilized polyacrylamide-sialyl Lewis A oligosaccharide, a non-limiting representative antigen of the invention.

Figure 8 is a schematic representation showing the inhibitory effect of sialyl Lewis A antigen, a non-limiting representative antigen of the invention, on the binding of Iodine-125 radiolabeled HB1 (also called ALT-4) antibody, a non-limiting representative therapeutic antibody of the invention, to immobilized CA19-9 antigen, a non-limiting antigen of the invention. Radio-immunoassay plates were coated with CA19-9 at 65 U/mL and incubated with radiolabeled HB1 at 0.33 μg/mL (a non-saturating amount) and increasing concentrations of Sialyl Lewis A antigen (0-100 μg/ml).

Figure 9 is a schematic representation showing Scatchard analysis of the binding of Iodine-125 radiolabeled HB1 mAb (also called ALT-4), a non-limiting representative therapeutic monoclonal antibody of the invention, to CA19-9 antigen, a non-limiting representative antigen of the invention. Radioimmunoassay strips were coated with CA19-9 antigen at 65 U/mL, and radiolabeled antibody was added to strips at different dilutions ranging from 0.0005 μg/mL to 10.5 μg/mL. The affinity of HB1 antibody to CA19-9 was determined by measuring the radioactivity of free and bound fractions in individual wells by Gamma counter.

Figures 10A, 10B and 10C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the caliper method. Eight measurements were performed, and the results are represented as AUC percent change in effect. Figures 10A, 10B and 10C represent the same data, illustrated as a bar graph (Fig. 10A), a column scatter (Fig. 10B), and a whicker box (Fig. 10C).

Figures 11A, 11B, and 11C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 11A), a column scatter (Fig. 11B), and a whicker box (Fig. 11C).

Figures 12A, 12B, and 12C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the water displacement method. Eight measurements were performed, and the results are represented as AUC percent change in effect. Figures 12A, 12B and 12C represent the same data, illustrated as a bar graph (Fig. 12A), a column scatter (Fig. 12B), and a whicker box (Fig. 12C).

Figures 13A, 13B, and 13C are schematic representations showing the inflammatory response in different rat groups for Example I as measured by the water displacement method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 13A), a column scatter (Fig. 13B), and a whicker box (Fig. 13C).

Figure 14 is a schematic representation of a column scatter graph showing the RAMA response with the inflammatory response in different rat groups for Example I. Figs. 10B and 12B are reiterated below Fig. 14 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 10B) or the water displacement method (Fig. 12B) with the level of immune response (Fig. 14)

Figures 15A, 15B and 15C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by caliper method. Eight measurements were performed, and the results are represented as AUC percent change. Figures 15A, 15B and 15C represent the same data, illustrated as a bar graph (Fig. 15A), a column scatter (Fig. 15B), and a whicker box (Fig. 15C).

Figures 16A, 16B, and 16C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 16A), a column scatter (Fig. 16B), and a whicker box (Fig. 16C).

Figures 17A, 17B, and 17C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by water displacement method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 17A), a column scatter (Fig. 17B), and a whicker box (Fig. 17C).

Figures 18A, 18B, and 18C are schematic representations showing the inflammatory response in different rat groups for Example II as measured by water displacement method.

Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 18A), a column scatter (Fig. 18B), and a whicker box (Fig. 18C).

Figure 19 is a schematic representation of a column scatter graph showing the RAMA response with the inflammatory response in different rat groups for Example II. Figs. 15B and 17B are reiterated below Fig. 19 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 15B) or the water displacement method (Fig. 17B) with the level of immune response (Fig. 19)

Figures 20A-20H are schematic representations of line graphs showing the correlation between the RAMA response and the inflammatory response at different time measurements in the group of rats treated with HB1 antibody, a non-limiting representative therapeutic antibody of the invention, in Example II. The inflammatory response was measured at 1.5 hours (Fig. 20A), 2.5 hours (Fig. 20B), 3.5 hours (Fig. 20C), 4.5 hours (Fig. 20D), 5.5 hours (Fig. 20E), 6.5 hours (Fig. 20F), or 7.5 hours (Fig. 20G) after injection of carrageenan. In Fig. 20H, the AUC percent change in effect was plotted against the RAMA (Ab2) response.

Figures 21A, 21B and 21C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the caliper method. Seven measurements were performed, and the results are represented as AUC percent change in effect. Figures 21A, 21B, and 21C represent the same data, illustrated as a bar graph (Fig. 21A), a column scatter (Fig. 21B), and a whicker box (Fig. 21C).

Figures 22A, 22B, and 22C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the caliper method. Seven measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 22A), a column scatter (Fig. 22B), and a whicker box (Fig. 22C).

Figures 23A, 23B, and 23C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the water displacement method. Seven measurements were performed, and the results are represented as AUC percent change in effect. Figures 23A, 23B, and 23C represent the same data, illustrated as a bar graph (Fig. 23A), a column scatter (Fig. 23B), and a whicker box (Fig. 23C).

Figures 24A, 24B, and 24C are schematic representations showing the inflammatory response in different rat groups for Example III as measured by the water displacement

method. Seven measurements were performed, and the results are represented as percent change in maximum effect. Figures 24A, 24B and 24C represent the same data, illustrated as a bar graph (Fig. 24A), a column scatter (Fig. 24B), and a whicker box (Fig. 24C).

Figure 25 is a schematic representation of a column scatter graph showing the RAMA response with the inflammatory response in different rat groups for Example III. Figures 21B and 23B are reiterated below Figure 20 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 21B) or the water displacement method (Fig. 23B) with the level of immune response (Fig. 25)

Figures 26A-26G are schematic representations showing the correlation between the RAMA response and the inflammatory response at different time measurements in the group of rats treated with HB1 antibody, a non-limiting representative therapeutic antibody of the invention, in Example III. The inflammatory response was measured at 1.5 hours (Fig. 26A), 2.5 hours (Fig. 26B), 3.5 hours (Fig. 26C), 4.5 hours (Fig. 26D), 5.5 hours (Fig. 26E), 6.5 hours (Fig. 26F), or 7.5 hours (Fig. 26G) after injection of carrageenan.

Figures 27A-27G are schematic representations showing the correlation between the RAMA response and the inflammatory response at different time measurements in the group of rats treated with F5 antibody, a non-limiting representative antibody of the invention, in Example III. The inflammatory response was measured at 1.5 hours (Fig. 27A), 2.5 hours (Fig. 27B), 3.5 hours (Fig. 27C), 4.5 hours (Fig. 27D), 5.5 hours (Fig. 27E), 6.5 hours (Fig. 27F), or 7.5 hours (Fig. 27G) after injection of carrageenan.

Figures 28A, 28B and 28C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by caliper method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 28A), a column scatter (Fig. 28B), and a whicker box (Fig. 28C).

Figures 29A, 29B, and 29C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 29A), a column scatter (Fig. 29B), and a whicker box (Fig. 29C).

Figures 30A, 30B, and 30C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by water displacement method.

Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 30A), a column scatter (Fig. 30B), and a whicker box (Fig. 30C).

Figures 31A, 31B, and 31C are schematic representations showing the inflammatory response in different rat groups for Example IV as measured by water displacement method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 31A), a column scatter (Fig. 31B), and a whicker box (Fig. 31C).

Figure 32 is a schematic representation of a column scatter graph showing the RAMA (Ab2) response with the inflammatory response in different rat groups for Example IV. Figs. 28B and 30B are reiterated below Fig. 32 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 28B) or the water displacement method (Fig. 30B) with the level of immune response (Fig. 32)

Figures 33A, 33B and 33C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by caliper method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 33A), a column scatter (Fig. 33B), and a whicker box (Fig. 33C).

Figures 34A, 34B, and 34C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by caliper method. Eight measurements were performed, and the results are represented as percent change in maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 34A), a column scatter (Fig. 34B), and a whicker box (Fig. 34C).

Figures 35A, 35B, and 35C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by water displacement method. Eight measurements were performed, and the results are represented as AUC percent change. These figures represent the same data, illustrated as a bar graph (Fig. 35A), a column scatter (Fig. 35B), and a whicker box (Fig. 35C).

Figures 36A, 36B, and 36C are schematic representations showing the inflammatory response in different rat groups for Example V as measured by water displacement method. Eight measurements were performed, and the results are represented as percent change in

maximum effect. These figures represent the same data, illustrated as a bar graph (Fig. 36A), a column scatter (Fig. 36B), and a whicker box (Fig. 36C).

Figure 37 is a schematic representation of a column scatter graph showing the RAMA (Ab2) response with the inflammatory response in different rat groups for Example V. Figs. 33B and 35B are reiterated below Figure 37 to allow comparison the of the extent of the inflammatory response as measured by the caliper method (Fig. 33B) or the water displacement method (Fig. 35B) with the level of immune response (Fig. 37)

Figures 38A-38H are schematic representations of histograms showing the binding of therapeutic monoclonal antibodies to human neutrophils as determined by single step indirect immunofluorescence flow cytometry (FACS). In Figs. 38A and 38B, colo 205 cells, which are known to express the Sialyl Lewis A epitope, were used as a positive control. Figs. 38C-38H are neutrophils stained with control (no primary antibody) (Fig. 38C), ALT-4 IgM antibody (Fig. 38D), ALT-3 IgG3 antibody (Fig. 38E), 7C2C5C12 IgM antibody (Fig. 38F), F5 IgM antibody (Fig. 38G), and Flopc-21 IgG3 antibody (Fig. 38H).

Figures 39A and 39B are schematic representations showing the instrumental setup and the schematic diagram of the *in vitro* inhibition of leukocyte rolling and adhesion in microchips channels coated with selectins.

Figure 40 is a schematic representation of a bar graph showing the inhibition of human polymorphonuclear leukocyte (PMNL) rolling in microchip channels coated with recombinant E-selectin, a non-limiting representative adhesion molecule of the invention, by the various indicated agents. The graph bars show the percentage of human PMNL rolling.

Figure 41 is a schematic representation showing the inhibition of human PMNL rolling in microchip channels coated with recombinant P-selectin, a non-limiting representative adhesion molecule of the invention, by the various indicated agents. The graph bars show the percentage of human PMNL rolling.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have developed methods and therapeutic compositions for reducing inflammation. The methods and therapeutic compositions of the invention are useful as analytical tools and as therapeutic tools. The invention also provides methods and therapeutic compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects. Standard reference works setting forth the general principles of the technology described herein include Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1994; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, New York, 1989; Kaufman *et al.* (Eds.), Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton, 1995; and McPherson, Ed., Directed Mutagenesis: A Practical Approach, IRL Press, Oxford, 1991. Standard reference works setting for the general principles of immunology and inflammation include Gallin *et al.*, Inflammation: Basic Principles and Clinical Correlates, Raven Press, New York, 1988; Kuby, J., Immunology, 3rd ed., W.H. Freeman, New York, 1997; Coligan *et al.* (Eds.), Current Protocols in in Immunology, John Wiley & Sons, New York, 1991; and Hurley, J.V., Acute Inflammation, 2nd ed., Churchill Livingstone, New York, 1983.

The published patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, published foreign patent applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. Any inconsistency between these publications and the present disclosure shall be resolved in favor of the present disclosure.

In a first aspect, the invention provides a method for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition, comprising administering to the animal a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the binding agent is administered with a pharmaceutically-acceptable carrier.

In a second aspect, the invention provides a therapeutic composition for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition, comprising a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent. Preferably, the composition further comprises a pharmaceutically-acceptable carrier.

In a third aspect, the invention provides a therapeutic composition comprising an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount. Preferably, the composition further comprises a pharmaceutically-acceptable carrier.

In a fourth aspect, the invention provides a method for reducing an immune condition in an animal suffering from cancer or inflammation or predisposed to suffer from cancer and inflammation, comprising administering to the animal a therapeutically effective amount of an agent that is capable of reducing inflammation and reducing cancer in an animal.

As used herein, by "agent" is meant a molecule of any size which may be naturally occurring or synthetic. Thus, an agent includes, without limitation, a chemical, a protein, a carbohydrate, a lipid, a nucleic acid, an acid, a base, a synthetic polymer, and a resin.

As used herein, by "immune condition" is meant cancer and/or inflammation. By "predisposed to suffer from an immune condition " is meant an animal that has a genetically or environmentally caused predisposition to suffer from an immune condition, but is not yet suffering from the immune condition. For example, the animal may have a certain type of MHC haplotype rendering it more susceptible to develop an immune condition, such as rheumatoid arthritis, but has not yet developed the condition. Another example is an animal which has been exposed to intense sunlight, but has not yet developed a sunburn. Another example is an animal which has been exposed to a carcinogen, but has not yet developed cancer.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the immune condition is cancer. In accordance with the invention, by "cancer" is meant the excessive growth of an abnormal cell in an animal resulting in the migration of metastatic

cells via the blood stream from the original anatomical site of the original abnormal cell to other parts in the body. In accordance with the invention, the detection of a secondary site of cancer caused by the migration of a metastatic cell is not required for an animal to be diagnosed as suffering from cancer. Rather, an animal (*e.g.*, a human) may have a cell growth biopsied. Upon a physician's inspection of the biopsied tissue and an assessment that the biopsied cells are likely malignant (*i.e.*, capable of metastasis), the animal may be treated in accordance with the invention.

One non-limiting cancer of the invention is melanoma. Other non-limiting cancers of the invention include breast cancer, colorectal cancer, bladder cancer, leukemia, lymphoma, cervix cancer, prostate cancer, testicular cancer, liver cancer, lung cancer, ovarian cancer, and pancreatic cancer.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the immune condition is inflammation. In accordance with the invention, by "inflammation" is meant a local response to cellular injury that is marked by any or all of capillary dilatation, leukocyte infiltration, redness, heat, and pain and that serves as a mechanism initiating the elimination of the inflammation-inducing antigen and of damaged tissue. The term includes acute inflammation, chronic inflammation, and excessive inflammation, such as that leading to inflammatory disease.

Acute inflammation exhibits a rapid onset and is of short duration. The characteristic signs of an acute localized inflammatory response generally include swelling, redness, heat, pain and loss of function (Kuby, J., Immunology, 3rd ed., W.H. Freeman, New York, pages 361-377, 1997). Infiltration of leukocytes into the tissue peaks within the first 6 hours of an acute inflammatory response, and most of them disappear from the inflamed area within 24-48 hours (Kuby, J., *supra*).

Chronic inflammation develops during persistence of an antigen due to infection or various pathologic conditions. The characteristic hallmark of chronic inflammation is the formation of granuloma. Many different types of cells may be found in the extravascular tissues in areas of chronic inflammation, including any or all of neutrophils, eosinophils, macrophages, epithelioid cells, plasma cells, lymphocytes, and fibroblasts (Hurley, J.V., Acute Inflammation, 2nd ed., Churchill Livingstone, New York, pages 135-144, 1983).

Excessive inflammation, either secondary to abnormal recognition of host tissue as "foreign" or deviation from an otherwise normal inflammatory response, leads to

inflammatory disease (Gallin *et al.*, Inflammation: Basic Principles and Clinical Correlates, Raven Press, New York, 1988). Most forms of acute and chronic inflammation are amplified as well as propagated as a result of the recruitment of humoral and cellular response (Gallin *et al.*, *supra*).

As used herein, by "reducing an immune condition" is meant the ability of an agent (e.g., a binding agent) of the invention to alleviate the symptoms of and/or prevent the onset of an immune condition in an animal suffering from the immune condition (or predisposed to suffer from the immune condition (*e.g.*, an animal exposed to the sun which has not yet developed a sunburn; an animal who has been exposed to a carcinogen which has not yet developed cancer; or an animal genetically predisposed to develop an immune condition which has not yet developed the immune condition)) when administered to that animal. Preferably, the alleviation of symptoms and/or prevention of onset is reduced in a treated animal as compared to the immune condition in an animal to which an agent of the invention has not been administered. Preferably, the reduction in the immune condition in an animal to which has been administered a binding agent of the invention is at least 10% lower as compared to an untreated animal; more preferably, the reduction is at least 25% lower; still more preferably, the reduction is at least 50% lower; even more preferably, the reduction is at least 75% lower; and most preferably, the reduction in the immune condition in an animal to which has been administered a binding agent of the invention is at least 100% lower as compared to an untreated animal (*i.e.*, an animal to which a binding agent of the invention has not been administered). Methods for determining the amount of an immune condition in an animal, where the condition is inflammation, include, without limitation, visual detection of redness and measurement of swelling by the caliper method or by the water displacement method described below. Methods for determining the amount of an immune condition in an animal, where the condition is cancer, include, without limitation, detection of the presence of a metastatic cancer cell and detection of cancer cell-induced angiogenesis.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the animal suffering from an immune condition is preferably a domesticated animal including, without limitation, domesticated fowl (*e.g.*, ducks, geese, chickens, turkeys, Cornish hens, and ostriches), domesticated livestock (*e.g.*, cattle, llamas, elephants, camels, pigs, and sheep), domesticated pets (*e.g.*, horses, cats, dogs, ferrets, hamsters, and guinea pigs); and laboratory animals (*e.g.*, primates (*e.g.*, baboons, Rhesus monkeys) and rodents (*e.g.*, mice, rats)).

In a preferred embodiment of the first, second, third, and fourth aspects of the invention, the animal suffering from the immune condition is a mammal. In a preferred embodiment of the first and second aspects of the invention, the animal suffering from the immune condition is a human.

As used herein, by the term, "induces an immune response" is meant that a cellular and/or humoral immune response is induced or increased in an animal to which has been administered a binding agent of the invention, such that the immune response is directed toward the administered binding agent. Thus, in preferred embodiments of the first, second, third, and fourth aspects of the invention, the immune response comprises both a humoral and a cellular immune response.

The presence of such an immune response can be detected by any of the well known immune response detection methods including, without limitation, detection of the presence of a T cell (*e.g.*, a cytotoxic T cell or helper T cell) whose T cell receptor specifically binds to the binding agent and/or to the adhesion molecule (in the context of the major histocompatibility complex), detection of the presence of a B cell whose B cell receptor (*i.e.*, surface-expressed IgM) specifically binds to the binding agent and/or to the adhesion molecule, and detection of the presence of an antibody (of any serotype) which specifically binds to the binding agent and/or to the adhesion molecule.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the immune response results in the generation of an antibody that specifically binds to the binding agent. Such an antibody that specifically binds to the binding agent is generated as a result of an immune response in the treated animal to the administered binding agent. One non-limiting theory explaining such an immune response is the idiotypic cascade first described by Jerne (Jerne, N.K., *Annals of Immunology* **125C**: 373-389, 1974). This theory, as illustrated for an idiiotypic cascade of antibodies, where the first antibody specifically binds to a tumor associated antigen, is illustrated on Figure 2 (Kingsbury *et al.*, *Leukemia* **12**: 982-991, 1998).

Adapting this theory to the present invention, an administered binding agent (*e.g.*, an antibody) that specifically binds to an adhesion molecule is designated Ab1. Ab1 reacts with epitopic determinants on the adhesion molecule. The animal to which has been administered a binding agent of the invention generates an immune response to that binding agent. Where the immune response comprises antibodies that bind to the binding agent, these antibodies are

designated Ab2. Some Ab2 antibodies (namely the Ab2β, recognize the adhesion molecule-binding site of Ab1 and resemble the original adhesion molecule epitope recognized by Ab1. Other Ab2 antibodies recognize sites present elsewhere on the variable region, and may (Ab2γ) or may not (Ab2α) interfere with the binding site (Steinitz *et al.*, *Journal of Immunology* **141**: 3516-3522, 1988).

Furthermore, the idotypic network theory postulated that each Ab2 might serve as the antigen for an additional antibody (Ab3). This Ab3 antibody, like the original binding agent (Ab1) specifically binds to the adhesion molecule to which Ab1 specifically bound. Thus, in certain preferred embodiments of the first and second aspects of the invention, the immune response results in the generation of an antibody that specifically binds to the adhesion molecule.

In one non-limiting example, the injection of a selectin (*e.g.*, E-selectin) specific binding agent (Ab1) into an animal suffering from an immune condition, where the immune condition is inflammation, could inhibit leukocyte (*i.e.*, white blood cell) rolling on the endothelial cells lining the walls of a blood vessel through two different mechanisms. The first mechanism of this non-limiting example is dependent on the direct effect of Ab1 that will act to block the selectin on either the white blood cell surface or endothelial cell membrane, and make it unavailable for binding by a selectin ligand (*e.g.*, sialyl Lewis A)-bearing cell. The effect of Ab1 would be direct but unfortunately would also be short- term due to the rapid clearance of Ab1 from the circulation (Ab1 usually remained in the circulation for several hours or few days only). The second mechanism is dependent on the induction of anti-idiotypic (Ab2) and anti-anti-idiotypic (Ab3) Abs. Since Ab2 would mimic a selectin, it will inhibit through its binding to selectin molecules the interaction between selectins and their natural ligands. Ab3 will act as Ab1 and compete with the naturally existing carbohydrte selectin ligand in being bound by its selectin and as a result, inhibit the interaction between selectins and their ligands through competitive inhibition.

In another non-limiting example, the injection of a carbohydrate selectin ligand (*e.g.*, sialyl Lewis A) specific binding agent (Ab1) into an animal suffering from an immune condition, where the immune condition is cancer, could inhibit metastatic cancer cell (*e.g.*, melanoma cell) rolling on the endothelial cells lining the walls of a blood vessel through two different mechanisms. The first mechanism is dependent on the direct effect of Ab1 that will act to block selectin ligand on either white blood cell surface or endothelial cell membrane, and make it unavailable for binding by a selectin-bearing cell. The direct effect of Ab1

would be direct but short- term. The second mechanism is dependent on the induction of anti-idiotypic (Ab2) antibodies which mimic the selectin ligand and anti-anti-idiotypic (Ab3) antibodies which mimic the Ab1 antibody that specifically bound to the selectin ligand. Ab2 would mimic a selectin ligand, thus inhibiting cancer cell rolling on endothelial cells and extravasation through its binding to selectin molecules. Ab3 will inhibit cancer cell rolling and extravasaion by competing with the naturally existing selectin molecule in binding to carbohydrate selectin ligand.

The effect of Ab2 and Ab3 (*i.e.*, the indirect effect of Ab1) would likely to be more efficient than the direct effect of Ab1 for the inhibition of leukocyte or cancer cell migration, since Ab2 and Ab3 antibodies are produced by the treated animal and remain in the circulation for a considerable period of time (usually several months).

As used herein, the term "therapeutically effective amount" is used to denote a dosage of binding agent effective to reduce an immune condition in the treated animal and/or a dosage of binding agent effective to induce an immune response to the binding agent in the treated animal. Preferably, such administration should be intravenous, intra-arterial, subcutaneous, parenteral, or transdermal. Preferably, a binding agent of the invention is administered at a dosage of less than about 8 mg per 30 kg body weight of the animal to be treated; preferably less than about 5 mg per 30 kg body weight; preferably less than about 3 mg per 30 kg body weight; still more preferably from about 0.5 mg to about 2 mg per 30 kg body weight; still more preferably from about 0.5 mg to about 1.5 mg per 30 kg body weight; and most preferably, a binding agent of the invention is administered at a dosage of about 1 mg per 30 kg body weight of the animal to be treated.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that, when administered to an animal, does not induce antibody-dependent cellular cytotoxicity (ADCC) in the treated animal. In certain preferred embodiments of the first and second aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that is the maximum amount of binding agent that, when administered to an animal, does not induce antibody-dependent cellular cytotoxicity (ADCC) in the treated animal. In these embodiments, ADCC may be assessed by incubating ^{51}Cr-labeled tumor cells with a binding agent according to the invention and adding fresh human peripheral blood mononuclear cells (PBMCs), followed by incubation for four hours and then

measurement of specific lysis (as determined by ^{51}Cr release). ADCC is absent if specific lysis is less than 15%.

In certain embodiments of the first, second, third, and fourth aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that, when administered to an animal, does not induce antibody-mediated toxicity in the treated animal. In certain preferred embodiments of the first and second aspects of the invention, the therapeutically effective amount of a binding agent is a dosage of the binding agent that is the maximum amount of binding agent that, when administered to an animal, does not induce antibody-mediated toxicity in the treated animal. By "antibody-mediated toxicity" is meant clinical toxicity, such as abnormal serum chemistries, impaired renal function, signs and symptoms of serum sickness, or anaphylaxis. Antibody-mediated toxicity is well-known to immunologist and described, for example, in Abbas *et al.*, Cellular and Molecular Immunology, 3rd ed., W. B. Saunders Co., Philadelphia, PA 1997 (see particularly pages 425-434). In certain embodiments, a single such dosage will therapeutically treat the animal. In other embodiments, the treatment may be ongoing, *e.g.*, administration of a dosage of a binding agent four times a year for three or more years. In one example, the administration of a dosage of a binding agent will be performed one injection per month for about three months to years.

In accordance with the invention, the term, "blood-borne cell," means any cell that can be carried by the blood stream. Thus, the definition includes bone marrow derived cells such as red blood cells and white blood cells which include, without limitation, lymphocytes (both T and B), natural killer cells, neutrophils (also called polymorphonuclear leukocytes or PMNL), eosinophils, basophils, monocytes, megakaryocytes, and platelets. Also included as blood-borne cells are metastatic cancer cells which have migrated (or are attempting to migrate) from the site of the original abnormal cell.

Thus, in those embodiments of the first, second, third, and fourth aspects of the invention, where the immune condition is inflammation, the blood-borne cell is a white blood cell. In certain embodiments, the white blood cell is, without limitation, a lymphocyte, a platelet, a granulocyte (*i.e.*, a a neutrophil, eosinophil, or a basophil), or a monocyte.

In those embodiments of the first, second, third, and fourth aspects of the invention, where the immune condition is cancer, the blood-borne cell is a cancer cell. Any cancer cell that is carried by the blood stream is included in the invention. In certain embodiments, the

cancer cell is, without limitation, a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

As used herein, by "adhesion molecule" is meant a molecule which is expressed on the surface of or released by either a blood-borne cell or a cell (*e.g.*, an endothelial cell) that lines a blood vessel and which can form an association with another adhesion molecule expressed on the surface of either a blood-borne cell or a cell lining a blood vessel. By "released by" means an adhesion molecule that is either cleaved from the cell surface of the indicated cell, secreted by the indicated cell, or released from the indicated cell upon lysis of that cell. Preferably, an adhesion molecule of the invention is a molecule expressed on the surface of or released by either on a blood-borne cell or an endothelial cell lining a blood vessel that forms an association with a second adhesion molecule on a blood-borne cell or an endothelial cell lining a blood vessel, such that association formed is between a blood-borne cell-expressed or released adhesion molecule and an endothelial cell-expressed or released adhesion molecule. Preferably, an adhesion molecule of the invention is one that is involved in the extravasion of white blood cells or cancer cells from the blood into the surrounding tissue. Preferably, the definition of "adhesion molecule" excludes the B cell receptor, the T cell receptor, and/or the signalling molecules comprising the B cell receptor complex (*e.g.*, Ig-α) and the T cell receptor complex (*e.g.*, CD3). The site on an adhesion molecule of the invention which is specifically bound by the binding agent is called an "epitope."

Non-limiting examples of adhesion molecules include the molecules which are members of the integrin family of proteins including, without limitation, LFA-1 (CD11a/CD18), VLA-4 (CD49d/CD29), and Mac-1 (CD11b/CD18). Adhesion molecules also include those molecules which are members of the immunoglobulin gene family of proteins including, without limitation, ICAM-1, B7, CD4, and CD28. Adhesion molecules also include those molecules which are members of the selectin family of molecules including, without limitation, P-selectin ELAM-1, CD62E), E-selectin (ELAM-1, CD62E) and L-selectin (CD62L) (Bevilacqua and Nelson, *Journal of Clinical Investigation* **91**: 370-387, 1993. Thus, in certain embodiments of the first and second aspects of the invention, the adhesion molecule is a selectin, such as P-selectin, E-selectin, or L-selectin.

Adhesion molecules of the invention also include glycoproteins, such as PSGL-1 (P-selectin glycoprotein ligand 1) and molecules (*e.g.*, mucins or mucoproteins) comprising carbohydrate epitopes, including the Sialyl Lewis A (SLe(a)) carbohydrate epitope and the Sialyl Lewis X (SLe(x)) carbohydrate epitope. Thus, in certain embodiments of the first and

second aspects of the invention, the adhesion molecule of the invention is a molecule comprising a carbohydrate epitope, such as a molecule comprising a Sialyl Lewis A epitope. Preferably, a molecule comprising a carbohydrate epitope is specifically bound by a selectin molecule.

In various embodiments of the third and fourth aspects of the invention, the agent is a binding agent that specifically binds to an adhesion molecule.

As used herein, by "binding agent" is meant a molecule or macromolecule that specifically binds to an adhesion molecule either in water, under physiological conditions (*e.g.*, in an animal's body), or under conditions which approximate physiological conditions with respect to ionic strength, *e.g.*, 140 mM NaCl, 5 mM $MgCl_2$. By "specifically binds" is meant a binding agent of the invention recognizes and forms a covalent association or, preferably, a non-covalent association with an adhesion molecule of the invention, but does not substantially recognize and form a covalent or non-covalent association with other molecules in a sample, *e.g.*, proteins that are not adhesion molecules. Likewise, an adhesion molecule of the invention bound by a binding agent of the invention that specifically binds to that adhesion molecule is said to be "specifically bound" by that binding agent. Preferably, a binding agent of the invention that specifically binds to an adhesion molecule of the invention forms an association with that adhesion molecule with an affinity of at least $10^6\ M^{-1}$, more preferably, at least $10^7\ M^{-1}$, even more preferably, at least $10^8\ M^{-1}$, and most preferably, at least $10^9\ M^{-1}$.

In certain embodiments, a binding agent of the invention is a peptide, a peptidomimetic, or a carbohydrate. By "peptide" is meant a molecule comprised of a linear array of two or more amino acid residues connected to each other in the linear array by peptide bonds. By "peptidomimetic" is meant a non-peptide molecule that mimics the structure and/or function of a peptide. By "carbohydrate or oligosaccharide" is meant a molecule comprising sugar residues. Non-limiting binding agents that are carbohydrates include SLe(x) oligosaccharide (Buerke *et al.*, *Journal of Clinical Investigation* **93**: 1140-1148, 1994; Lefer *et al.*, *Circulation* **90**: 2390-2401, 1994), and SLe(a) oligosaccharide.

Non-limiting binding agents of the invention include antibodies. Thus, in certain embodiments of the first, second, third, and fourth aspects of the invention, the binding agent is an antibody, such as a monoclonal antibody. In certain preferred embodiments, the binding agent is a murine monoclonal antibody.

For example, the monoclonal antibody of the invention, HB1 (also called ALT-4), is a murine IgM monoclonal antibody (mAb) secreted by the HB1 hybridoma cell line. HB1 specifically binds to the tumor marker, CA19-9, which comprises the carbohydrate epitope Sialyl Lewis A (Mukae *et al.*, *Am. Rev. Respir. Dis.* **148**(3): 744-751, 1993). Immunoassay analysis has revealed that HB1 also specifically binds to Sialyl Lewis A. HB1 hybridoma cells were maintained in standard media (RPMI-1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin, 10% v/v fetal bovine serum (FBS)).

Another non-limiting antibody of the invention is HB2 (also called ALT-3), a IgG3 murine monoclonal antibody secreted by the HB2 hybridoma cell line. HB2 specifically binds to the tumor marker CA19-9, and has also been shown to specifically bind to sialyl Lewis A. HB2 hybridoma cells were maintained in standard media.

Yet additional non-limiting binding agents that are antibodies include PB1.3, an anti-P selectin monoclonal antibody (Weiser *et al.*, *Shock* **5**(6): 402-407, 1996; Yamada *et al.*, *European Journal of Pharmacology* **346**: 222-224, 1998); ARP2-41, an anti-rat-P-selectin mAb (Tojo *et al.*, *Glycobiology* **6**(4): 463-9, 1996; Ohnishi *et al.* *Immunopharmacology* **34**: 161-170, 1996); BBIG-E5, an anti-E-selectin mAb (Altavilla *et al.*, *European Journal of Pharmacology* **270**: 45-51,1994); DREG-200, an anti-L-selectin mAb, (Ma *et al.*, *Circulation* **88**: 649-658, 1993); MEL-14, an anti-L-selectin mAb (MEL-14) (Pizcueta and Luscinskas, *American Journal of Pathology* **145**(2): 461-467, 1994); anti- SLe(x) mAb (Yoshinori *et al.*, *Journal of Pathology* **180**: 305-310, 1996); anti- SLe(x) mAb (anti-human and rat) (clone no. 2H5; commercially available from BD Pharmingen, San Jose, CA); anti- SLe(x) mAb (clone no. CSLEX; commercially available from Becton Dickinson Immunocytometry Systems, San Jose, CA); anti- SLe(x) mAb (clone no. CSLEX1; commercially available from Signet Laboratories, Inc., Dedham, MA); anti-PSGL-1 (CD162) antibodies (*e.g.*, commercially available from Biodesign Intl., Saco, ME (*e.g.*, clone no. 3E2.25.5(PL1) (Catalog No. P91090M) and clone no. 5D8.8.12 (PL2) (Catalog No. P91209M) and the various anti-SLe(a) mAb commercially available from Biodesign Intl., Saco, ME (*e.g.*. clone no. 241 (Catalog No. M37241B); clone no. 59 (95-330-07) (Catalog No. M37059M); clone no. 62 (Catalog No. M37062M); clone no. 67 (Catalog No. M37067M); clone no. 192 (Catalog No. M37192M); and clone no. 239 (Catalog No. M37239M)).

Yet additional non-limiting binding agents of the invention include the anti-human E-selectin and anti-human P-selectin antibodies commercially available from R & D Systems (Minneapolis, MN, USA).

In certain embodiments, the binding agents of the invention comprises a complementarity determining region (CDR) of an antibody or a T cell receptor that specifically binds to an adhesion molecule of the invention. By "complementarity determining region (CDR) of an antibody" is meant a portion of an antibody that specifically binds to an epitope, including any framework regions necessary for such binding, and which is preferably comprised of a subset of amino acid residues encoded by the immunoglobulin gene heavy chain V, D, and J regions, the immunoglobulin gene light chain V and J regions, and/or any combinations thereof. By "complementarity determining region (CDR) of a T cell receptor" is meant a portion of a T cell receptor that specifically binds to an epitope (*e.g.*, in context of the major histocompatibility complex), including any framework regions necessary for such binding, and which is preferably comprised of a subset of amino acid residues encoded by the T cell receptor β chain gene V, D, and J regions, the T cell receptor α chain gene V and J regions, and/or any combinations thereof. Preferably, the CDR of the invention is derived from a human or a murine antibody.

Given the numerous antibodies disclosed herein, one of skill in the art are enabled to make a variety of antibody derivatives, wherein such antibody derivatives are also included as binding agents of the invention. For example, Jones *et al.* (*Nature* **321**: 522-525, 1986) disclose replacing the CDRs of a human antibody with those from a murine antibody. Marx, J.L. (*Science* **229**(4712): 455-456, 1985) discusses chimeric antibodies having murine variable regions and human constant regions. Rodwell, J.D. (*Nature* **342**(6245): 99-100, 1989) discusses lower molecular weight recognition elements derived from antibody CDR information. Clackson, T.P. (*Br. J. Rheumatol.* **30 Suppl 2**:36-39, 1991) discusses genetically engineered monoclonal antibodies, including Fv fragment derivatives, single chain antibodies, fusion proteins, chimeric antibodies, and humanized rodent antibodies. Reichman *et al.* (*Nature* **332**: 323-327, 1988) discloses a human antibody on which rat hypervariable regions have been grafted. And Verhoeyen *et al.* (*Science* **239**: 1534-1536, 1988) teaches the grafting of a mouse antigen binding site onto a human antibody.

In addition, given the numerous antibodies disclosed herein, those of skill in the art are enabled to design and produce peptidomimetics having binding characteristics similar or superior to such complementarity determining regions, wherein such peptidomimetics are included as binding agents of the invention. Peptidomimetics are well known and described, for example, in Horwell, D.C., *Bioorg. Med. Chem.* **4**: 1573-1576, 1996; Liskamp *et al.*, *Recl.*

Trav. Chim. Pays-Bas. **1**: 113, 1994; Gante *et al.*, *Angew. Chem. Int. Ed. Engl.* **33**: 1699, 1994; and Seebach *et al.*, *Helv. Chim. Acta* **79**: 913, 1996.

The binding agents of the present invention are preferably purified binding agents. By "purified" is meant that a binding agent is at least 60%, preferably at least 75%, even more preferably at least 90%, and most preferably at least 95% free from contaminants, such as endotoxins.

In a non-limiting example, where the binding agents of the invention are monoclonal antibodies, to purify the antibodies (*e.g.*, HB1 and HB2), the antibodies were purified from ascites. For ascites preparation, adult mice (age 6 weeks) were primed by injecting 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid) into the peritoneum (i.p.). Two weeks later the mice were injected i.p., with 5 x 10^6 hybridoma cells (*e.g.*, HB1 or HB2) resuspended in 0.5 ml of PBS. Ascetic fluid was tapped from mice 2 to 3 times in due time, and the collected fluid was incubated for 1 hour at room temperature and transferred to 4°C overnight. The fluid was centrifuged at 2000 rpm for 15 minutes and the supernatant was stored at –20 °C.

The monoclonal antibodies were then purified and concentrated. As various methods for purifying and concentrating antibodies are known to those of skill in the art, the following purification methods are non-limiting examples of methods that can be used for purifying antibodies from ascites. It should be noted that these methods can also be used to purify and concentrate antibodies from the supernatant of hybridoma cells grown *in vitro* under standard tissue culture conditions.

For mannan binding protein (MBPP affinity chromatography purification, the ascetic fluid containing the IgM was dialyzed against binding buffer (10 Mm Tris, 1.25 M NaCl, 20 mM $CaCl_2$, and 0.02 % Sodium azide (NaN_3) at a pH of 7.4), after which the ascetic sample was diluted 1: 1 v/v with binding buffer, and filtered with 0.22 mm filter. The MBP column (commercially available from Pierce, Rockford, IL, USA) was prewashed with 2 column volume (20 ml) of elution buffer (10 mM Tris, 1.25 M NaCl, 2mM EDTA and 0.02% NaN_3), and then equilibrated with 4 column volume (40 ml) of binding buffer. The ascetic fluid (maximum 1.5 ml/ 5 ml of gel) was applied to the column, allowed to completely enter the gel, and incubated at 4 °C for 30 minutes. The column was washed with 9 column volumes of the binding buffer to remove the unbound proteins. The wash was monitored for the presence of proteins by measuring the absorbance at 280 nm, using the binding buffer as a reference. The column was removed from the cold and incubated with the elution buffer at room

temperature for 1 hour, after which the column was washed with the elution buffer, and minimum of 14 fractions were collected each with a volume of 3 ml. The elution of IgM was monitored for the presence of proteins by spectrophotometer at 280 nm using the elution buffer as a reference. At the completion of the purification the column was washed with 2 column volume of deionized water and then by 2 column volumes of 4 °C binding buffer and stored at 4 °C.

The euglobulin precipitation method is a non-chromatographic method used for the purification of both murine IgG_3 and IgM mAbs, which takes advantage of their euglobulin properties. This method was performed as essentially described by (Garcia-Gonzalez et al., *Journal of Immunological Methods* **111**: 17-23, 1988). Briefly, $CaCl_2$ was added to the ascetic fluid (final concentration, 25 mM) to generate fibrin formation. When the clot was formed, it was removed by paper filtration. Then the filtered ascetic fluid was dialyzed for 2 hours at 20 °C (IgG3), or for 15 hours at 4 °C (IgM) against 100x volume of demineralized water (pH 5.5). The ascetic fluid was centrifuged in a BECKMAN L8-55 ultracentrifuge at 22,000 x g for 30 minutes and the precipitate was recovered and suspended in 1M NaCl/0.1 M Tris-HCl pH 8. Dialysis and precipitation were repeated twice. Purified pellet with a high lipid content was mixed with 1.7 M NaCl and centrifuged for 3 hours at 27,000 x g. Lipid supernatant was discarded, the clarified mAbs solution was dialyzed against 0.1M Tris HCl, 1M NaCl pH 8, centrifuged at 22,000 x g for 30 minutes. The purified pellet was suspended in phosphate buffered saline (PBS).

For Protein A affinity chromatography purification, a 5 ml Protein A column (commercially available from Pierce, Rockford, IL, USA) was washed with 2 column volumes of elution buffer (0.1 mM Glycine, 1mM NaCl, 0.001 % tween 20, pH 4). Then the column was equilibrated with 5 column volumes of binding buffer (50 mM Tris–HCl, 1mM NaCl, 0.001 % tween 20, pH 8). The ascetic sample containing IgG_3 antibody was diluted 1:1 v/v with binding buffer and loaded to the column at a rate of 0.75 ml/ minute, then the column was washed with 50 ml of binding buffer, at a rate of 1.5 ml / minute. The collected fractions containing unbound proteins were checked by spectrophotometer at 280 nm, using the binding buffer as a reference. The column was washed with 15 ml of elution buffer. Then the eluted fractions were collected at a rate of 1 ml /minute and adjusted to a pH of 7 using 50 mM Tris-HCl. Elution of bound proteins was monitored by spectrophotometer at 280 nm, using the elution buffer as a reference.

For removal of endotoxins from purified immunoglobulins, a 10 ml Detoxi-Gel column (commercially available from Pierce, Rockford, IL, USA) was regenerated by washing the gel with 5 column volumes of 1% sodium deoxycholate. Then the column was washed with 3 column volumes of pyrogen-free water. The purified and concentrated immunoglobulin was loaded to the column, and then the sample was collected by gravity flow using PBS as elution buffer.

Before use, the ultrafilter YM-30 membrane (MW cutoff 30,000 Dalton) (commercially available from Fisher Scientific, Nepean, ON, Canada) was floated with distilled water for one hour, changing the water three times. Then the membrane was mounted in an ultrafiltration cell and rinsed with distilled water at 20 psi (3.7 atm) for at least 5 minutes. The samples of purified Igs were loaded in the cell, and the sample flow through the ultrafilter membrane was operated under a maximum pressure of 40 psi (4.7 atm). The antibody solution was concentrated approximately 10x and then washed at least three time using PBS.

The quantification of purified F5 IgM, 7C2C5C12 IgM and Flopc-21 IgG_3 mAbs was done by ELISA where plates were coated with goat anti-mouse Ig (commercially available from Sigma Chemical Co., St. Louis, MO, USA). Note that this ELISA was also used to detect the presence of either Ab2 antibody in a treated animal of the invention, or the presence of rat anti-mouse antibody, since all of the injected antibodies are murine antibodies injected into rats. A schematic diagram of this assay is shown on Figure 3.

The quantification of HB1 IgM and HB2 IgG_3 mAbs was done by ELISA where the plates were coated with CA19-9 antigen. Note that this ELISA was also used to detect the presence of Ab3 antibodies. A schematic diagram of this assay is shown on Figure 4.

For each tested mAb, an isotype matched control mAb was used as a standard. Clarified ascites containing the mouse IgG3k monoclonal antibody Flopc-21 was obtained from SIGMA Chemical Co. (St Louis, MO, USA). Control F5 antibody is murine IgM monoclonal antibody secreted by the F5 hybridoma cell line (commercially available from the American Type Culture Collection, Maryland, USA). Control 7C2C5C12 antibody is a murine IgM monoclonal antibody which specifically binds to *Trichinella spiralis* and which is secreted by the 7C2C5C12 hybridoma cell line (commercially available from the American Type Culture Collection, Maryland, USA). Both F5 hybridoma cells and 7C2C5C12 hybridoma cells were maintained in standard media (RPMI-1640 supplemented with 2 mM

L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin, 10% v/v fetal bovine serum (FBS)).

For the ELISA assay for determining the concentration or the binding activity of mAbs, microtiter strips were coated with 100 ml of PBS containing either 2.5 μg/ml of goat anti-mouse Ig (whole molecule), 75 U/ml of CA19-9 antigen (commercially available from Altarex Corp., Edmonton, Alberta, Canada), or 2.5 μg/ml of polyacrylamide-SLe(a) (commercially available from Altarex Corp., Edmonton, Alberta, Canada) and incubated overnight at 4 °C. The coating solution was discarded and 150 μl of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS) was added to each well in order to block the non-specific binding sites on the wells. After one hour of incubation at room temperature the plates were washed three times with PBST. 100 μl of binding buffer containing mAbs was added in different dilutions. All wells were incubated for 1 hour at room temperature, followed by three washings with PBST (PBS containing 0.1% Tween-20, pH 7.1)). Then 100 μl of binding buffer containing goat anti-mouse Ig (H+L) -HRP labeled (commercially available from Southern Biotechnology Associates Inc., Birmingham, AL, USA) was added to each well at a dilution of 1/5000 v/v, incubated for 1 hour, and followed by three washings with PBST. The activity of bound mAbs was determined by adding a 100 μl of ABTS (Peroxidase solution B and ABTS peroxidase substrate, 1:1 v/v dilution) to each well (ABTS Peroxidase solution and ABTS peroxidase substrate commercially available from LifeTechnologies GIBCO BRL (Burlington, Ontario). The optical density was measured at dual wavelength, 405 and 492 nm using an ELISA reader (Titertek multiskan Plus MK II).

An SDS-polyacrylamide gel electrophoresis was used to check the purity of each of the purified mAbs. Ab samples were diluted 1:4 v/v with SDS-PAGE sample buffer (0.5 M Tris-HCl at pH 6.8, 20% glycerol, 10% w/v SDS, 10% b- mercaptoethanol and 0.5 % bromphenol blue) and heated for 4 minutes at 95 °C. The Tris-HCL ready gel sandwich (10% acrylamide) (commercially available from BioRad, Hercules, CA, USA) was inserted into the Mini-PROTEAN II cell clamp assembly and aligned properly. Then the gel sandwich was attached to the inner core of the Mini-PROTEAN II cell (commercially available from BioRad). The upper and the lower buffer chambers were filled with approximately 200 ml of 1x running buffer (0.3% Tris base, 1.45% glycine, 0.1% SDS, pH 8.3). 6 μl of molecular weight markers in sample buffer was applied to one of the wells, while the antibody samples, each with a volume of 30 μl (1.5-5 μg of antibody) were applied

to the rest of the wells in the gel. The core assembly was inserted into the lower buffer tank and the gel was run at 200 V constant voltage for approximately 45 minutes. The gel was then removed from the gel sandwich, immersed in staining solution (10% acetic acid, 0.025% Coomassie BlueG-250, methanol 40%) for 1.5 hour, and destained in destaining solution (10% acetic acid, 40% methanol) until the desired destaining was reached.

Antibodies of the invention were purified and then resolved by SDS-polyacrylamide gel electrophoresis. As shown in Figure 5, antibodies purified by the mannan binding protein affinity chromatograph (lanes 2 and 4), by the euglobin precipitation method (lanes 3 and 7), and by protein A affinity chromatography (lane 6) are "purified" for the purposes of the present invention (compare to lanes 5 and 8 for old samples of purified monoclonal antibodies.

In the non-limiting embodiment where the binding agent of the invention is a monoclonal antibody, the binding of the various murine monoclonal antibodies to tumor antigen CA19-9 and to sialyl Lewis A was determined. Using the ELISA assay described above and schematically depicted in Figure 4 (using CA19-9 coated plates), the various purified monoclonal antibodies were tested for binding to purified CA19-9. A range of mAb concentration from 0 to 0.64 mg/ml was used. As can be seen in Figure 6, for both HB1 (ALT-4) and HB2 (ATL-3) mAbs, even at a concentration of only 10 ng/ml, maximum binding to CA19-9 was observed which is likely to be due to the presence of multiple sialyl Lewis A epitopes within the CA19-9 antigen. As expected, the two control mAbs F5 and 7C2C5C12 did not bind to CA19-9 antigen. Surprisingly, however, the control mAb Flopc-21 that was used in the same concentration range bound to CA19-9 with an observed dose effect.

In order to determine more specifically the epitope recognized by HB1 and HB2, a second ELISA assay (as described above) was performed which used the carbohydrate antigen SLe(a) conjugated to polyacrylamide in order to carry a direct binding assay. As shown in Figure 7, both HB1 (ALT-4) and HB2 (ALT-3) mAbs recognized the carbohydrate epitope SLe(a). In addition, Figure 7 demonstrated that Flopc-21 mAb is directed against a different CA19-9 epitope determinant than the SLe(a) epitope recognized by HB2 and HB1 and, thus, does not interact with SLe(a) carbohydrate oligosaccharide.

The specificity of HB1 mAb for SLe(a) was also studied. To do this, HB1 was first radiolabeled with ^{125}I (iodinated). 50 μg of HB1 mAb was mixed with approximately 600

μCi of Na ^{125}I. 10 ml of 2.5 mg/ml of chloramine-T (CT) (commercially available from Sigma Chemical Co.) was added to the reaction vial and gently shaken for 30 seconds. Then, 10 μl of 5 mg/ml sodium metabisulfate (Na-Met) was added to the mixture and shaken gently for 15 seconds. Finally, 20 μl of 1M sodium iodide (NaI) was added and the whole mixture was gently shaken for 5 seconds. The reaction mixture was loaded to the Desalting Econo-pac 10 DG chromatographic column (commercially available from BioRad, Hercules, CA, USA) pre-washed three times with 0.5% BSA in PBS. The vial was rinsed with 100 μl of PBS and transferred to the column. The column was then washed with 0.5 ml PBS fractions 12 to 14 times. Each elute was collected separately in numbered tubes. The radioactivity of elutes was monitored by a Geiger counter. The fractions containing the mAbs were pooled, labeled, placed in lead containers, and stored at 4 °C. The quantification of ^{125}I labeled HB1 mAb in the pooled fractions was done by ELISA as described above (and depicted in Figure 4) using plates coated with CA19-9. A nonradioactive control HB1 mAb was used as standard.

For competitive inhibition assay, RIA strips coated with 50 μl of PBS containing a 65 U/ml of tumor antigen CA19-9 were incubated overnight at 4 °C. After discarding the coating solution, the non-specific binding sites were blocked with 150 μl/well of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS). The wells were incubated for 1 hour at room temperature, and then washed with PBST three times. Dilutions of SLe(a) antigen (commercially available from Oxford GlycoSciences Inc., MA, USA) were made serially in binding buffer (1% BSA, 0.02% thimerosal in PBS) from 0 to 100 μg / ml and mixed with iodinated HB1 mAb. A nonsaturating concentration (0.33 μg/ml) of iodinated HB1 (ALT-4) mAb was chosen in order to obtain a final count per minute (c.p.m.) concentration of approximately 40,000 c.p.m./well. 100 μl of each dilution was added to different wells and allowed to incubate for 1.5 hours. After that the supernatant was discarded, the strips were washed with PBST three times and broken down to individual wells and the radioactivity was measured (bound fraction).

As shown in Figure 8, only at high concentrations, namely 50 μg /ml and 100 μg/ml, was SLe(a) antigen able by competitive inhibition to reduce the binding of HB1 to CA19-9 antigen by 35% and 60% respectively. The requirement for such a high concentration of Sle^a to inhibit the binding of HB1 mAb to CA19-9 could be due to the fact that HB1 Ab is an IgM and therefore has 10 binding sites for each SLe(a) molecule. In addition, the affinity of HB1

Ab for the SLe(a) epitope carried by CA19-9 is likely to be higher than the affinity of HB1 Ab for soluble SLe(a). Such difference in affinity may be due to a more stabilized three-dimensional configuration of the SLe(a) carried by CA19-9 and/or to a longer sequence recognized by HB1 within CA19-9 molecule.

The affinity of HB1 (ALT-4) mAb to Ca19-9 antibody was next determined by scatchard analysis using iodinated HB1 mAb. To do this, radio-immunoassay (RIA) strips were coated with 100 µl of PBS containing 65 U/ml of CA19-9 antigen overnight at 4 °C. After discarding the coating solution, the nonspecific binding sites were blocked with 150 µl/well of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS). The wells were incubated for 1 hour at room temperature with gentle shaking, and then washed three times with PBST (PBS, 0.1% Tween-20, pH 7.1). Each strip was then incubated with a range of concentration from 0.005 to 10.5 µg/ml of I^{125} labeled HB1 antibody in 100 µl of binding buffer (1% BSA, 0.02% thimerosal in PBS) at room temperature for 1 hour. Then, 50 µl of the HB1 supernatant was taken from each well and the radioactivity was measured (free fraction) gamma counter. The strips were again washed three times with PBST, broken into individual wells and the radioactivity of each well was measured (bound fraction) by Gamma counter.

Thus, the affinity of iodinated HB1 antibody to CA19-9 antigen was determined by measuring the radioactivity of free and bound fractions in individual wells by Gamma counter. As shown in Figure 9, the Kd value from this experiment was calculated to be equal to 0.94 nM.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

Example I

Male Sprague Dawley (SD) rats age 6 weeks, weight 275-300 grams, were injected five times with 250 mg/rat of either keyhole limpet hemocyanin (KLH)-conjugated HB1 mAb, control KLH-conjugated F5 mAb, or were simply received PBS injection via intraperitoneal (i.p.) route.

Serum collection was routinely done prior to each immunization. The tails of rats were cut slightly at the tip and the blood was collected in serum separator microtainers. During this procedure the rats were anaesthetized with ethyl ether. The collected blood was centrifuged at 300 x g for five minutes; serum was separated, and used to test for the detection of RAMA and Ab3 responses. The animals were kept under care until inflammation was induced and measured.

The mAbs were conjugated to KLH in order to enhance their immunogenicity. Chemical conjugation is usually very efficient but is likely to decrease the binding activity of the mAbs. For each conjugation, we therefore determined the remaining binding activity of the KLH-mAbs. This was done by direct ELISA as described above and schematically depicted in Figure 4 using CA19-9 coated plates.

To conjugate antibodies with keyhole limpet hemocyanin (KLH), 1 mg of either HB1 mAb or F5 mAb (control) was suspended in PBS solution was mixed with 1 mg of KLH (commercially available from SIGMA Chemical Co., St Louis, MO, USA) and 2 ml of glutaraldehyde (final concentration, 25%). The mixture was incubated for 1 hour at room temperature and then 75 mg of glycine was added to the mixture. The mixture was stirred for an addition 1 hour and then dialyzed against distilled water for three days. The binding activity of the mAbs to CA19-9 antigen was measured before and after conjugation with KLH and compared by ELISA using CA19-9 antigen-coated plates.

Rat anti-mouse antibody (RAMA) and Ab3 responses were measured between each injection. The ELISA assays used for the measurement of Ab2 (RAMA) and Ab3 humoral responses are described above and illustrated in Figures 3 and 4, respectively.

The rats were then subjected to acute inflammation in the right hind paw by injection of carrageenan (carrageenan lambda type IV (gelatin, vegetable, Irish moss); commercially available from Sigma Chemical Co.) five days after the last injection of antibody. To induce inflammation in the rat right hind paw (intraplantary), 0.05 ml of 1% carrageenan in 0.9 % NaCl as injected into the rats five days after the last immunization with antibody (*e.g.*, KLH conjugated HB1, F5, or PBS only). The animals were lightly anaesthetized with ethyl ether for the injections and for inflammation measurements.

Paw edema was used as a characteristic sign of inflammation, and was quantitated at regular interval of time after carrageenan injection, with a total of 8 measurements. Paw edema was measured by two different methods. The caliper method was used to measure

paw thickness, and water displacement method to measure paw volume. The thickness of rat paw was measured in inches by a caliper device before and after carrageenan injection at desired time intervals. The paw volume was measured by immersing the paw up to the tibio-tarsae articulation in a cylinder filled with water, and the fluid volume replaced by immersed paw was measured before and after carrageenan injection at desired time intervals.

Both paw thickness and paw volume measurements of the inflammatory response results were analyzed in the same way. The measurements were plotted as area under the curve (AUC) percent change in effect and as percent change in maximum effect. "Percent change in effect" means the percent change in inflammatory response in each rat from time zero to each consecutive time of inflammation measurement. "AUC percent change in effect (area under the curve)" means the sum of the percent changes in inflammatory response from time zero to each consecutive time of inflammation measurement for each rat. The average of AUC percent change in effect was calculated per rat group. "Percent change in maximum effect" means the percent change in inflammatory response from time zero to the time where maximum inflammation has occurred in each rat.

Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 10A, 10B, and 10C; and as percent change in maximum effect in Figures 11A, 11B, and 11C. Paw volume measurement results are illustrated as AUC percent change in effect in figures 12A, 12B, and 12C; and as percent change in maximum effect in Figures 13A, 13B, and 13C.

For both paw thickness and paw volume measurements, the least edema was observed in HB1 group, but this difference in change in effect, measured by caliper method (P= 0.631) or by water displacement method (P= 0.655), was not statistically significant.

Ab3 was not detected in any of the rat groups tested. The results obtained for the measurement of the RAMA immune response are shown in Figure 14. The RAMA response was detected in only one rat in the group of rats treated with HB1 mAb (rat # 5, Figure 15). The PBS only-injected group did not receive any mAb injection and consequently had no RAMA response (Figure 14). The absorbance obtained by ELISA corresponds therefore to background level (Figure 14). The F5 control mAb-injected group showed absorbance values above background level. These values correspond to anti-isotypic and/or anti-allotypic immune responses (the assay performed with HB1 coated plates cannot measure the anti-idiotypic response specific for F5 antibody). The HB1 therapeutic mAb-injected group

showed absorbance values above background level that corresponds to either anti-isotypic, anti-idiotypic or anti-allotypic immune response or a combination of them, among which only the idiotypic response will have the therapeutic effect.

It is worth noting that in the HB1 group, one rat (rat # 5) had a high RAMA response (see Fig. 14). Of particular interest, this particular rat did not develop any inflammatory response (see Figures 10B and 12B).

Example II

Male Sprague Dawley rats (age six weeks) were injected four times with 500 µg/rat of either KLH-conjugated HB1, control KLH-conjugated F5, control KLH-conjugated 7C2C5C12 mAbs, or have simply received PBS injection via i.p. route.

RAMA and Ab3 responses were measured between each injection. Induction and measurement of inflammation were done by the same methods as described in Example I. Paw edema was observed and measured at intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as described in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figure 15A, 15B, and 15C; and as percent change in maximum effect in Figures 16A, 16B, and 16C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 17A, 17B, and 17C; and percent change in maximum effect in Figures 18A, 18B, and 18C.

For both paw thickness and paw volume measurements, the least edema was observed in HB1-injected group, but this difference in change in effect, measured by caliper method ($P= 0.134$) or by water displacement methods (0.073) was not statistically significant.

Ab3 was not detected in any of the rat groups tested. A very low RAMA immune response was detected in the control groups (F5 and 7C2C5C12). The HB1-injected group was determined using the method depicted in Figure 3 to be the only group with a high RAMA immune response (Figure 19). Despite the fact that the group treated with HB1 antibody had the highest immune response, no significant correlation was found between the RAMA response and the therapeutic efficacy as shown in Figure 19.

The inflammatory responses of the HB1 injected rats at different measurement times was potted as percent change in effect against the RAMA (Ab2) response. As shown in

Figures 20A-20H, the correlation between the therapeutic efficacy and RAMA in HB1 group was not found to be significant at any time of the inflammation measurements.

Example III

Male Sprague Dawley rats (age six weeks) were injected four times with 500 mg/rat of either HB1, control F5 mAbs mixed with QUIL A adjuvant (commercially available from Calbiochem, San Deigo, USA), or have simply received PBS injection via subcutaneous (s.c.) route. RAMA and Ab3 responses were measured between each injection at regular intervals. Induction and measurement of inflammation was done by the same methods as described in Example I. Paw edema was observed and measured at regular intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 21A, 21B, and 21C; and as percent change in maximum effect in Figures 22A, 22B, and 22C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 23A, 23B, and 23C; and as percent change in maximum effect in Figures 24A, 24B, and 24C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method (P= 0.253) or by water displacement method (P= 0.358) was not statistically significant.

Ab3 was not detected in any of the rat groups tested. RAMA immune response was detected in the therapeutic HB1 group, and to a lesser extent in the control F5 group as shown in Figure 25 using the method as depicted in Figure 3.

The extent of inflammatory response was correlated with the extent of the level of RAMA immune response in HB1 group as shown in Figures 26A-26G. Of particular interest is the demonstration in HB1 rat group of a good correlation between RAMA immune response and the inflammatory response, *i.e.* the higher the RAMA immune response, the lower the inflammatory response. Surprisingly, some correlation was also observed between the extent of the inflammatory response and the RAMA response in the group treated with F5 mAb as shown in Figures 27A- 27G.

Example IV

Male Sprague Dawley rats (age six weeks) were injected four times with 500 μg/rat of either HB1 mAb or have simply received PBS injection via intravenous (i.v.) route. RAMA and Ab3 responses were measured between each injection at regular intervals. Induction and measurement of inflammation was done by the same methods as in experiment 1. Paw edema was observed and measured at regular intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 28A, 28B, and 28C; and as percent change in maximum effect in Figures 29A, 29B, and 29C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 30A, 30B, and 30C; and as percent change in maximum effect in Figures 31A, 31B, and 31C.

For both paw thickness and paw volume measurements, the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method (P= 0.111) or by water displacement method (P= 0.188) was not statistically significant.

Ab3 was not detected in any of the rat groups tested.

As shown in Figure 32, the RAMA immune response was detected (using the method depicted in Figure 3) in one rat that belonged to the therapeutic HB1 group. AUC percent change in effect from the measurements of caliper and water displacement methods showed that one rat in the therapeutic HB1 group had responded with minimal inflammatory response as shown in Figures 28B and 30B, respectively. Therefore, the extent of the inflammatory response as shown in Figures 28B and 30B was compared to the level of RAMA immune response in each rat in Figure 32. In fact, the same rat that showed the highest RAMA immune response as shown in Figure 32 also showed the least inflammatory response measured by both caliper and water displacement methods as shown in Figures 28B and 30B, respectively.

Example V

Rats were injected four times with 500 mg/rat of either KLH-conjugated HB2, control KLH-conjugated Flopc-21 mAbs, or have simply received PBS injection via i.p. route. RAMA and Ab3 immune responses were measured between each injection. Induction and

measurement of inflammation were done by the same methods as described in Example I. Paw edema was observed and measured at regular intervals of time.

Paw thickness and paw volume measurement results were analyzed and plotted as described in Example I. Paw thickness measurement results are illustrated as AUC percent change in effect in Figures 33A, 33B, and 33C; and as percent change in maximum effect in Figures 34A, 34B, and 34C. Paw volume measurement results are illustrated as AUC percent change in effect in Figures 35A, 35B, and 35C; and as percent change in maximum effect in Figures 36A, 36B, and 36C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method (P=0.074) or by water displacement method (P= 0.065) was not statistically significant.

Ab3 was not detected in any of the rat groups tested in Example V. Using the method depicted in Figure 3, all tested groups showed minimal (background) RAMA immune response as shown in Figure 37.

Example VI

The binding of HB1 (ALT-4), HB2 (ALT-3), and control monoclonal antibodies F5 (IgM), 7C2C5C12 (IgM), and Flopc-21 (IgG3) to PMNLs and lymphocytes was quantitated by FACScan and fluorescent microscopy. The binding of HB1, HB2, and control mAbs to HUV-EC-C cells was also examined by fluorescent microscopy.

Human umbilical vein endothelial cells (HUV-EC-C) is an endothelial cell line derived from the vein of a normal, human umbilical cord, obtained from American Type Culture Collection (Maryland, USA). HUV-EC-C cells were maintained in MCDB 131 media supplemented with 10 ng/ ml of HEGF, 12 mg/ ml of BBE, 1 mg/ ml of hydrocortisone, 5 % v/v FBS, 0.05 mg/ ml of Gentamycin sulfate, and 0.05 mg/ ml of amphotericin B.

To isolate human white blood cells, 6 ml of Histopaque –Ficoll solution was added to a 15 ml test tube, and then a 6-ml of heparin-treated fresh human blood was carefully layered on top of the gradient. Tubes were centrifuged at 1700 rpm (450 x g) for 35 minutes at room temperature. The band at the upper interface (mononuclear cells) was retrieved (5 ml) and mixed with 10 ml of 0.45% NaCl and 20 ml of PBS (8 mM Di-sodium hydrogen

orthophosphate, 3 mM potassium dihydrogen orthophosphate, 0.14 M NaCl). The tubes were centrifuged at 1500 rpm (400 g) for 15 minutes at 21 °C. The pellet was retrieved and washed three times with 10 ml of 3 mM EDTA, 1% FBS in PBS solution without magnesium and without calcium and centrifuged at 800 rpm (250 x g) for 10 minutes. The pellet was suspended in 2 ml of RPMI + 5% fetal bovine serum (FBS). In the case of isolating human PMNL, the band at the lower interface was retrieved and treated in the same way as with monouclear cells. The purified cell pellet (mononuclear or PMNL cells) was suspended at 1x 10^7 cells/ml in RPMI media, 5% FBS and kept at 37 °C until used. Cell viability was determined by exclusion of 0.4% trypan blue.

For FACS analysis, $1x10^6$ white blood cells or HUV-EC-C washed in PBS, 1% bovine serum albumin (BSA), were incubated with primary antibody (*i.e.*, HB1, HB2, F5, 7C2C5C12, or Floppc-21) at a concentration of 5 μg/ ml in PBS, 1% BSA. The incubation was performed at 4 °C and after 45 minutes the cells were washed three times with PBS, 1% BSA. The cells were then incubated with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse antibody at 4 °C for 45 minutes and washed three times. The stained cells were resuspended in PBS, 1% BSA. Fluorescence associated with the cells was analyzed either by FACScan flow cytometry (Becton Dickinson, Mountain View, CA) or by fluorescence microscopy. As negative control cells were incubated according to the same procedure but without primary antibody.

Examples of histograms obtained by FACS analysis are shown in Figures 38A-38H. Colo 205 cells, which were used as a positive control (Figs. 38A and 38B), are known to express the Sialyl Lewis A epitope on their surface. Colo 205 cells are a human adenocarcinoma cell line derived from metastatic colon cancer that produces CA19-9 antigen. Colo 205 cells were obtained from the American Type Culture Collection (Maryland, USA), and were maintained in standard media (RPMI-1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin, 10% v/v fetal bovine serum (FBS)).

As can be seen in Figures 38C-38H, all tested mAbs did not show significant binding to human neutrophils. None of the antibodies tested showed significant binding to rat neutrophils nor to HUVEC cells.

Example VII

The *in vitro* inhibition of leukocyte rolling on microchips by the therapeutic antibodies of the invention was next determined. For these studies, a microchip glass wafer device was used that consists of channels in which in vitro cell rolling and adhesion can be observed under flow conditions, the process that mimics the *in vivo* cell rolling and adhesion events.

To investigate the rolling and adhesion of cells, a 10 cm x 10 cm glass wafer with a series of simple "Y" mixer flow manifolds was used in this assay. The "Y" mixer flow manifold consisted of 50 microns in depth and 300 microns in width channels with a flow region downstream. These channels are structured in similar way to capillaries and therefore, the use of such channels allows the observation of cell rolling and adhesion in vitro that mimics the *in vivo* cell rolling and adhesion events. The microchip was secured into an aluminum microchip holder, and the microchip reservoirs were glued onto the microchip with 5-minute epoxy resin. The channels were conditioned with concentrated nitric acid (HNO_3), 2 M of sulfuric acid (H_2SO_4) and 1 M of sodium hydroxide (NaOH) (10 minutes each) before and after each experiment. The channels were then coated with 20 µg/ml of recombinant human E-selectin or P-selectin (commercially available from R & D Systems, Minneapolis, MN, USA) for 2 hours at 37 °C to mimic the surface of a vessel wall endothelial lining. A microsyringe pump that operated as a negative pressure source was used to drive the cells through the channels at a flow rate of 2.5 ml/min for the 50 micron deep device. This flow rate ensured a high enough shear rate (800 s^{-1}) to mimic blood flow in a vascular capillary. The channels were monitored using a Reichert Microstar (25X 280 x 220 mm field of view) and JVC color CCD camera mounted on the microscope (see Figure 39A). The experiments were recorded onto tapes with a JV VHS recorder.

A 1x 10^7 cells/ml solution of human PMNL or lymphocytes (isolated as described in Example VI) suspended in RPMI, 5% FBS was pumped to the channel through an injection route and allowed to run at an optimum flow rate for the purpose of achieving the rolling step. Since each channel had two sides, one side of each channel was used to test the agent under study and the other side of the channel was used as a control for cell rolling only without the addition of any agents (see Figure 39B). The cell suspensions added to different sides of the channel were regulated by a luminal fluidic flow that allowed these cell suspensions to continue running at different sides of the channel and prevented cell diffusion from one side of the channel to the other.

The following agents were tested at a volume of 10 µl and at a dilution of 1:10 v/v in RPMI and 5% FBS: fresh human serum (negative control), fresh rat serum, HB1 rat serum; and F5 rat serum. 10 µl of 100 µg/ml of anti-human E-selectin antibody (commercially available from R & D Systems, Minneapolis, MN, USA) was used as positive control for E-selectin coated channels. Serum of rats immunized with HB1 monoclonal antibody from Example III was obtained from rats who showed a high RAMA response (rat # 2, 4, 5, 6, 7, and 8, collectively). Serum of rats immunized with F5 monoclonal antibody from Example III was obtained from rats who showed a high RAMA response (rat # 4, 6, and 7, collectively). Once the rolling step was achieved, 10 µl of each of the above mentioned agent was added to different channels. The pump sucked the added sample into the channel to start the inhibition step. The images of cell rolling and inhibition steps were captured at different times and the number of cells was counted in both sides of the channel. Non-adherent cells appeared as streaks on the video image. The cell counts were conducted on a PC computer installed with a perception video board and image Pro Plus software.

The same agents were also tested on P-selectin coated channels under the same conditions as with E-selectin coated channels. For P-selectin coated channels, anti-human P-selectin antibody (commercially available from R & D Systems, Minneapolis, MN, USA) was used as a positive control.

Since there was no consistent rolling of rat leukocytes on the recombinant human E- or P-selectin coated channels and since rat E- and P-selectins were not available, the assay was carried only with human leukocytes (and not with rat leukocytes). The images of cell rolling and inhibition steps were captured at different times and the rolling cells were counted on both sides of the channel (see Figures 39A and 39B). The effect of each of the tested agents was determined as the percentage of rolling human PMNL from those in the control channel (100%) in which no agents were added.

As shown in Figures 40 and 41, respectively, normal human serum did not cause a reduction in human PMNL rolling on human E-selectin or human P-selectin coated channels. The same experiment performed with normal rat serum instead of normal human serum revealed by microscopic observation that such treatment resulted in an increased adherence of a small number of leukocytes on both E- and P- selectin coated channels. This effect however was very mild and did not affect the rolling of cells on either E- or P- selectin coated channels as shown in Figures 40 and 41, respectively.

Anti-E- and anti P-selectin mAbs were used to demonstrate effective rolling inhibition of PMNL on E-selectin (Fig. 40) and P- selectin (Fig. 41) coated channels, respectively. There was only 1.4 % of rolling cells observed with the addition of anti-E- selectin mAb, as compared to the side with the control rolling cells as shown in Figure 40, and 14.4% rolling cells with the addition of anti-P-selectin mAb as shown in Figure 41. Therefore, the rolling of human PMNL leukocytes on recombinant human E- and P- selectin coated channel was inhibited significantly by anti-E- and anti-P- selectin mAbs ("+ve control" in Figs. 40 and 41).

It was of particular importance to observe that the addition of 1:10 dilution of the RAMA positive serum obtained from rats immunized with HB1 antibody (rat # 2, 4, 5, 6, 7 and 8 collectively, Example III) caused a significant inhibitory effect on PMNL rolling on both human E-selectin and human P- selectin coated channels ("ALT-4 rat serum Ab2 +" in Figs. 41 and 42, respectively). In fact, the HB1 rat serum (*i.e.*, the ALT-4 rat serum from rats who showed a positive RAMA response) was able to reduce the rolling of PMNL on E-selectin coated channels to 0% resulting in a 100 % inhibition ($P< 0.001$) (see Fig. 40). The same rat serum resulted in a 76 % inhibitory effect on P-selectin coated channels ($P< 0.001$), since it was able to reduce the rolling of PMNL on P-selectin coated channels from 100 % to 23.2 % (see Fig. 41). In opposition to normal serum, no change in cell adherence or cell morphology was observed with the addition of rat serum immunized with HB1 antibody.

Unexpectedly, the RAMA positive control F5 rat serum also showed some inhibition of the rolling of PMNL leukocytes on recombinant human E- and P-selectin coated channels ("F5 rat serum Ab2+" in Figs. 40 and 41). However, this inhibition was considerably less marked than the one observed with the therapeutic monoclonal antibody HB1. The addition of serum obtained from rats immunized with F5 antibody who were positive for RAMA response (rats # 4, 6 and 7 collectively, Example III) caused a 45 % reduction in PMNL rolling on E-selectin coated channels ($P< 0.001$); and a 33 % reduction in PMNL rolling on P-selectin coated channels ($P< 0.001$) as shown in Figures 40 and 41 respectively.

Example VIII

A rodent model of melanoma is used to determine the effect of the binding agents of the invention in reducing melanoma. A murine melanoma cell line, such as clone B16 (commercially available as Catalog No. CRL-6322 from the American Type Culture

Collection, Manassas, VA) is injected subcutaneously or intra-peritoneally into syngeneic mice (*e.g.*, C57BL/6J mice) or immunodeficient nude mice (both commercially available from The Jackson Laboratory, Bar Harbor, ME) at an established primary site in an amount sufficient for the melanoma cells to establish tumor growth at the primary tumor site in the mice. A first set of mice is injected, and the time needed for metastasis of the melanoma cells to occur is recorded.

The experimental set of rats is injected with the melanoma cells. At the time metastasis is known to occur based on the results obtained from the first set of mice, the experimental set is divided into different groups of animals, each of which is administered a therapeutically effective amount of the various binding agents of the invention including, without limitation, HB1, HB2, ARP2-41, DREG-200, MEL-14, BBIG-E5, and anti-sialyl Lewis X antibodies via a subcutaneous, intraperitoneal, or intravenous injection. Control antibodies include 7C2C5C12 and F5.

Mice injected with binding agents of the invention that specifically bind to adhesion molecules expressed either on the cell surface of the melanoma cells or on the cell surface of endothelial cells lining blood vessel walls are found to have a reduced number of tumor formation at secondary sites (*i.e.*, sites distinct from the primary tumor site) than those mice injected with the control antibodies.

CLAIMS

1. A method for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition, comprising administering to the animal a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent.

2. The method of claim 1, wherein the adhesion molecule is a molecule comprising a carbohydrate epitope.

3. The method of claim 2, wherein the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A.

4. The method of claim 1, wherein the adhesion molecule is a selectin.

5. The method of claim 4, wherein the selectin is P-selectin or E-selectin.

6. The method of claim 1, wherein the immune condition is inflammation.

7. The method of claim 6, wherein the blood-borne cell is a white blood cell.

8. The method of claim 7, wherein the white blood cell is selected from the group consisting of a lymphocyte, a platelet, a granulocyte, and a monocyte.

9. The method of claim 1, wherein the immune condition is cancer.

10. The method of claim 9, wherein the blood-borne cell is a cancer cell.

11. The method of claim 10, wherein the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

12. The method of claim 1, wherein the binding agent is an antibody.

13. The method of claim 12, wherein the antibody is a monoclonal antibody.

14. The method of claim 1, wherein the immune response results in the generation of an antibody that specifically binds to the binding agent.

15. The method of claim 1, wherein the immune response results in the generation of an antibody that specifically binds to the adhesion molecule.

16. The method of claim 1, wherein the immune response comprises a humoral and a cellular immune response.

17. The method of claim 1, wherein the animal is a domesticated animal.

18. The method of claim 1, wherein the animal is a human.

19. The method of claim 1, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal.

20. The method of claim 1, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal.

21. The method of claim 1, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

22. A composition for reducing an immune condition in an animal suffering from the immune condition or predisposed to suffer from the immune condition comprising a therapeutically effective amount of a binding agent that specifically binds to an adhesion molecule expressed on the surface of or secreted by a blood-borne cell or expressed on the surface of or released by an endothelial cell lining a blood vessel, wherein the binding agent induces an immune response in the animal to the binding agent.

23. The composition of claim 22, wherein the adhesion molecule is a molecule comprising a carbohydrate epitope.

24. The composition of claim 23, wherein the carbohydrate epitope is Sialyl Lewis X or Sialyl Lewis A.

25. The composition of claim 22, wherein the adhesion molecule is a selectin.

26. The composition of claim 20, wherein the selectin is P-selectin or E-selectin.

27. The composition of claim 22, wherein the immune condition is inflammation.

28. The composition of claim 28, wherein the blood-borne cell is a white blood cell.

29. The composition of claim 28, wherein the white blood cell is selected from the group consisting of a lymphocyte, a platelet, a granulocyte, and a monocyte.

30. The composition of claim 22, wherein the immune condition is cancer.

31. The composition of claim 30, wherein the blood-borne cell is a cancer cell.

32. The composition of claim 31, wherein the cancer cell is selected from the group consisting of a breast cancer cell, a melanoma cell, a liver cancer cell, a lung cancer cell, a leukemic cell, and a lymphoma cell.

33. The composition of claim 22, wherein the binding agent is an antibody.

34. The composition of claim 33, wherein the antibody is a monoclonal antibody.

35. The composition of claim 22, wherein the immune response results in the generation of an antibody that specifically binds to the binding agent.

36. The composition of claim 22, wherein the immune response results in the generation of an antibody that specifically binds to the adhesion molecule.

37. The composition of claim 22, wherein the immune response comprises a humoral and a cellular immune response.

38. The composition of claim 22, wherein the animal is a domesticated animal.

39. The composition of claim 22, wherein the animal is a human.

40. The composition of claim 22, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-dependent cellular cytotoxicity (ADCC) in the animal.

41. The composition of claim 22, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that does not induce antibody-mediated toxicity in the animal.

42. The composition of claim 22, wherein the therapeutically effective amount of the binding agent is a dosage of the binding agent that is less than about 8 mg per 30 kg body weight of the animal.

43. The composition of claim 22, further comprising a pharmaceutically-acceptable carrier.

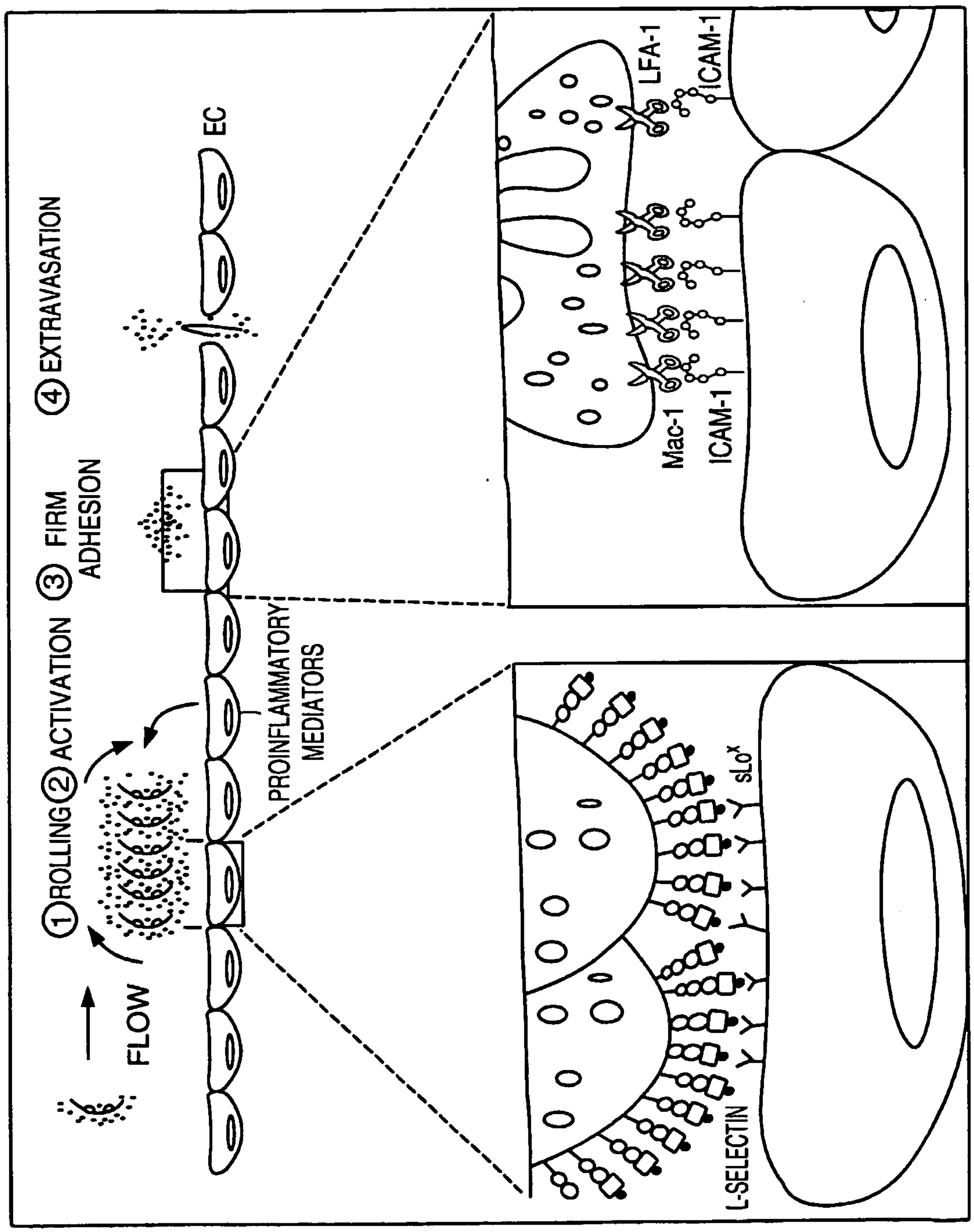
44. A therapeutic composition comprising an agent that is capable of reducing inflammation and reducing cancer in an animal when administered to the animal in a therapeutically effective amount.

45. The composition of claim 44, wherein the agent is a binding agent that specifically binds to an adhesion molecule.

46. A method for reducing an immune condition in an animal suffering from cancer or inflammation or predisposed to suffer from cancer and inflammation, comprising administering to the animal a therapeutically effective amount of an agent that is capable of reducing inflammation and reducing cancer in an animal

47. The method of claim 46, wherein the agent is a binding agent that specifically binds to an adhesion molecule.

FIG. 1


FLOW
1 ROLLING
2 ACTIVATION
3 FIRM ADHESION
4 EXTRAVASATION
EC
PROINFLAMMATORY MEDIATORS
L-SELECTIN
sLe^x
Mac-1
ICAM-1
LFA-1
ICAM-1

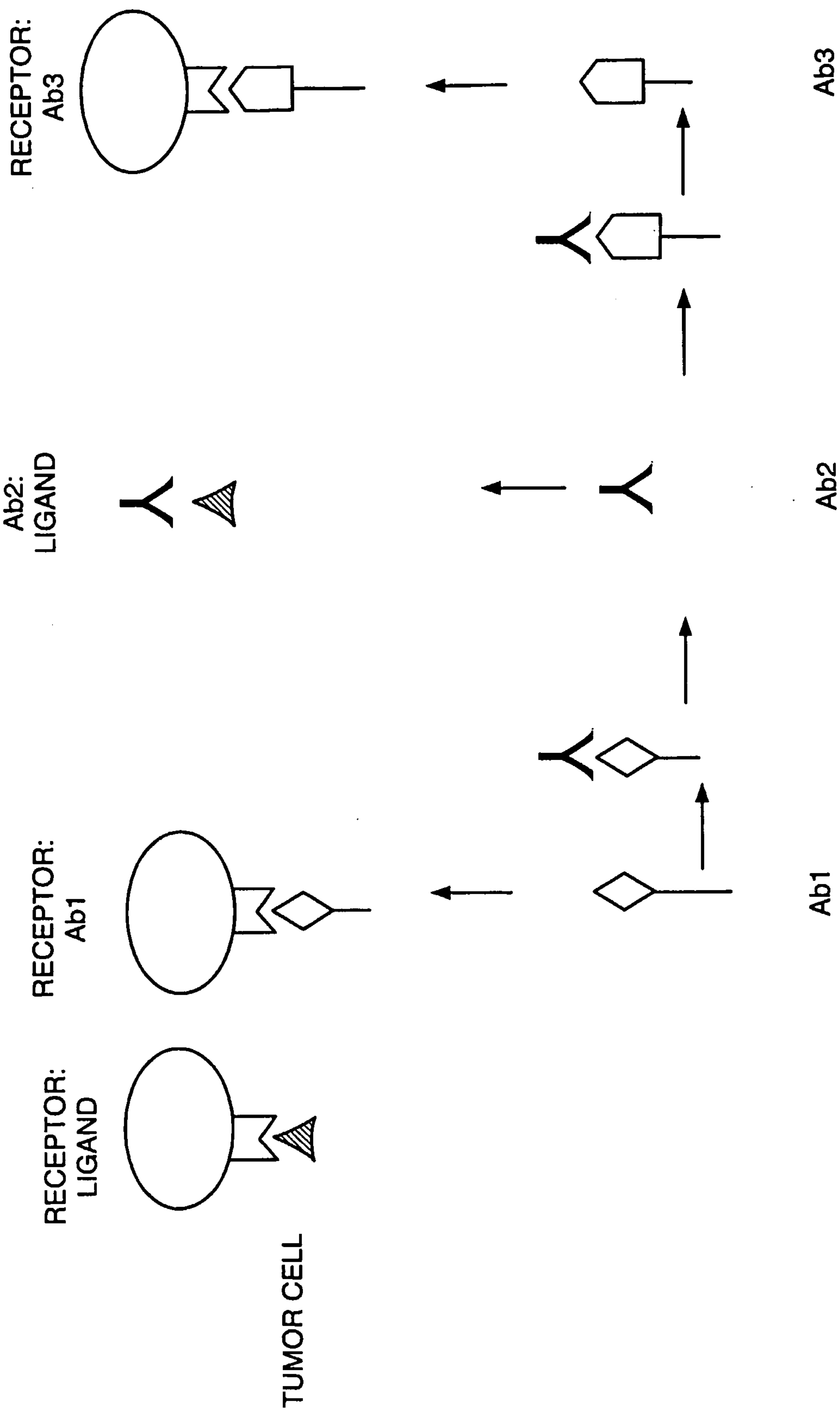

RECEPTOR: Ab3
Ab2: LIGAND
RECEPTOR: Ab1
RECEPTOR: LIGAND
TUMOR CELL
Ab3
Ab2
Ab1

FIG. 2

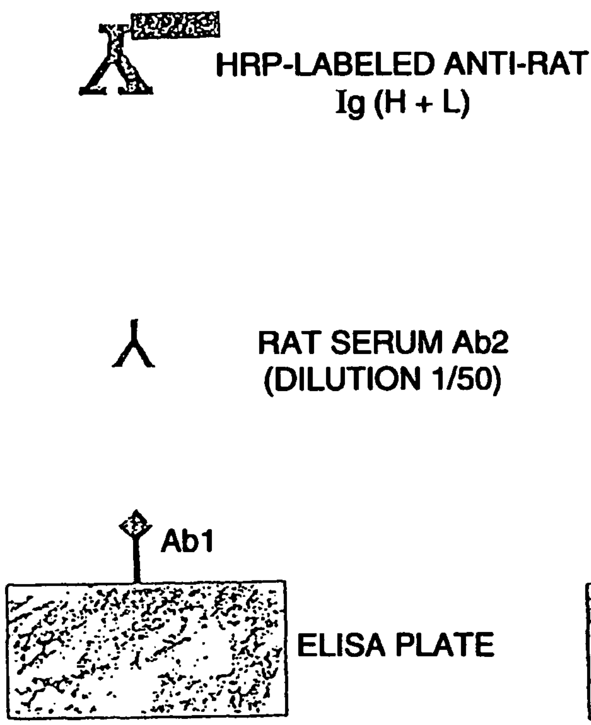

HRP-LABELED ANTI-RAT
Ig (H + L)
RAT SERUM Ab2
(DILUTION 1/50)
Ab1
ELISA PLATE

FIG. 3

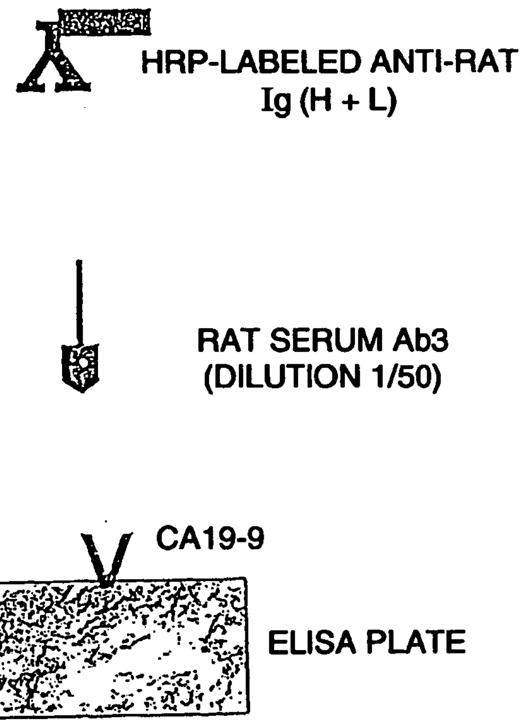

HRP-LABELED ANTI-RAT
Ig (H + L)
RAT SERUM Ab3
(DILUTION 1/50)
CA19-9
ELISA PLATE

FIG. 4

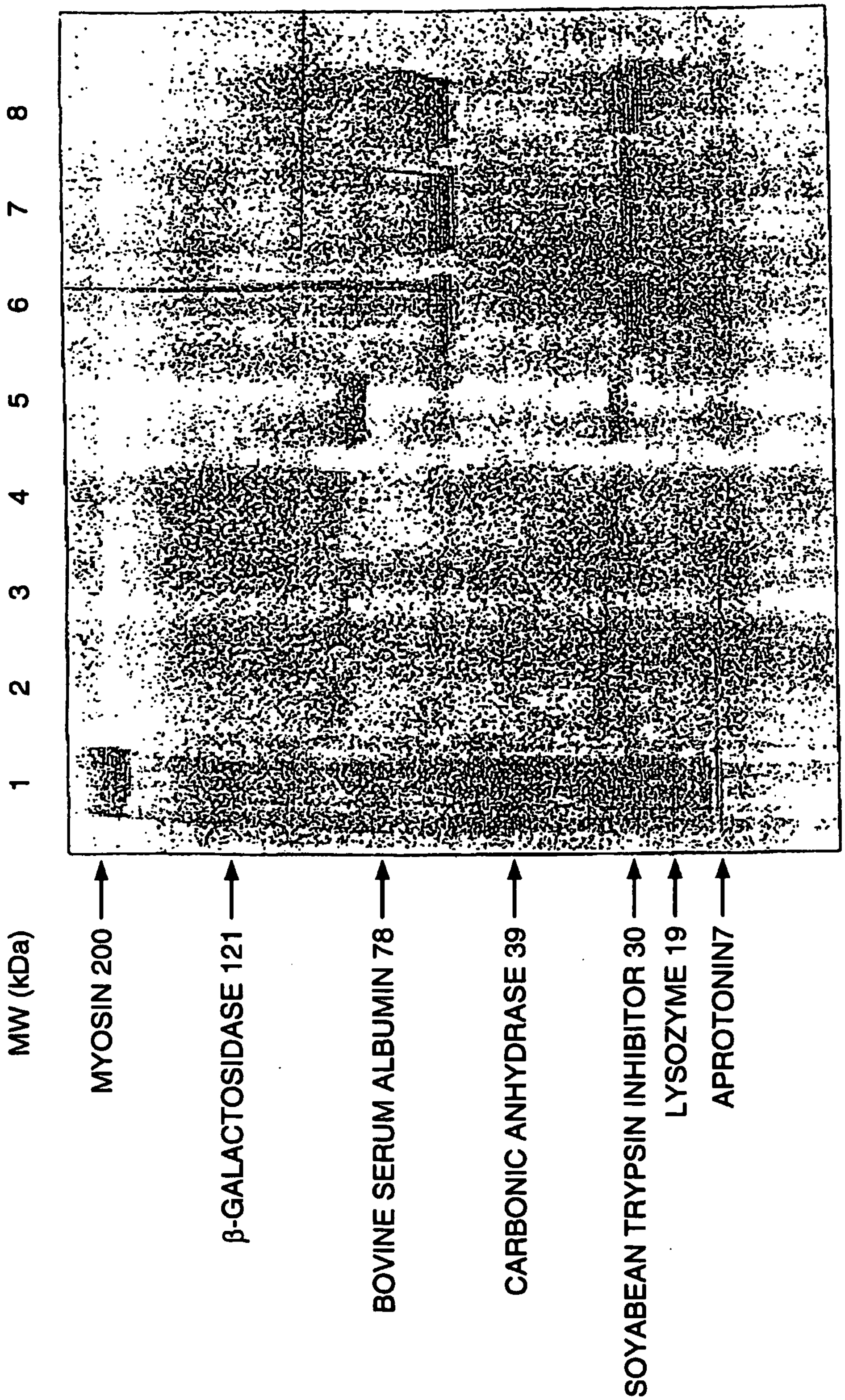

1
2
3
4
5
6
7
8
MW (kDa)
MYOSIN 200
β-GALACTOSIDASE 121
BOVINE SERUM ALBUMIN 78
CARBONIC ANHYDRASE 39
SOYABEAN TRYPSIN INHIBITOR 30
LYSOZYME 19
APROTONIN7

FIG. 5

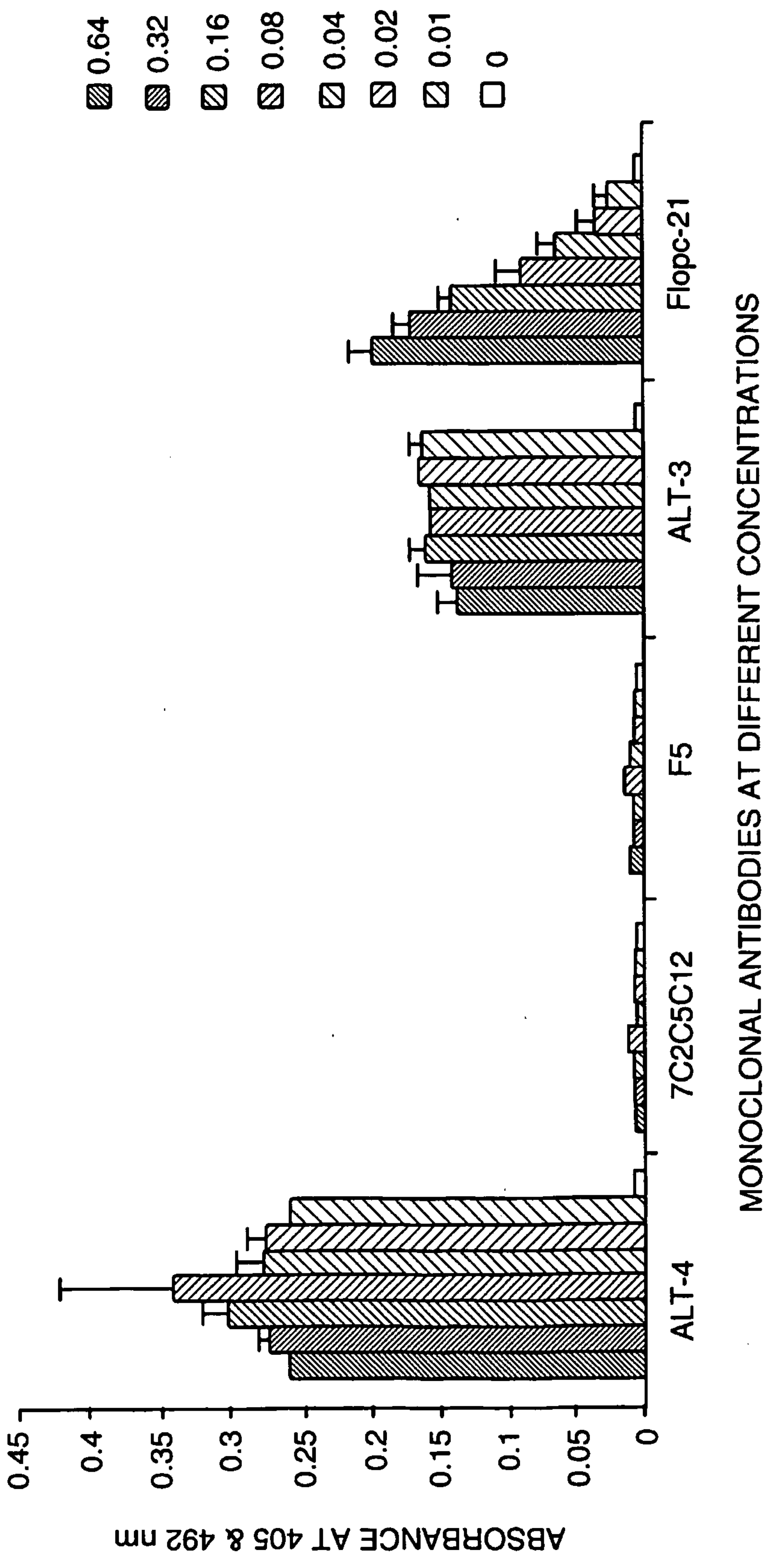

0.64
0.32
0.16
0.08
0.04
0.02
0.01
0
ABSORBANCE AT 405 & 492 nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ALT-4
7C2C5C12
F5
ALT-3
Flopc-21
MONOCLONAL ANTIBODIES AT DIFFERENT CONCENTRATIONS

FIG. 6

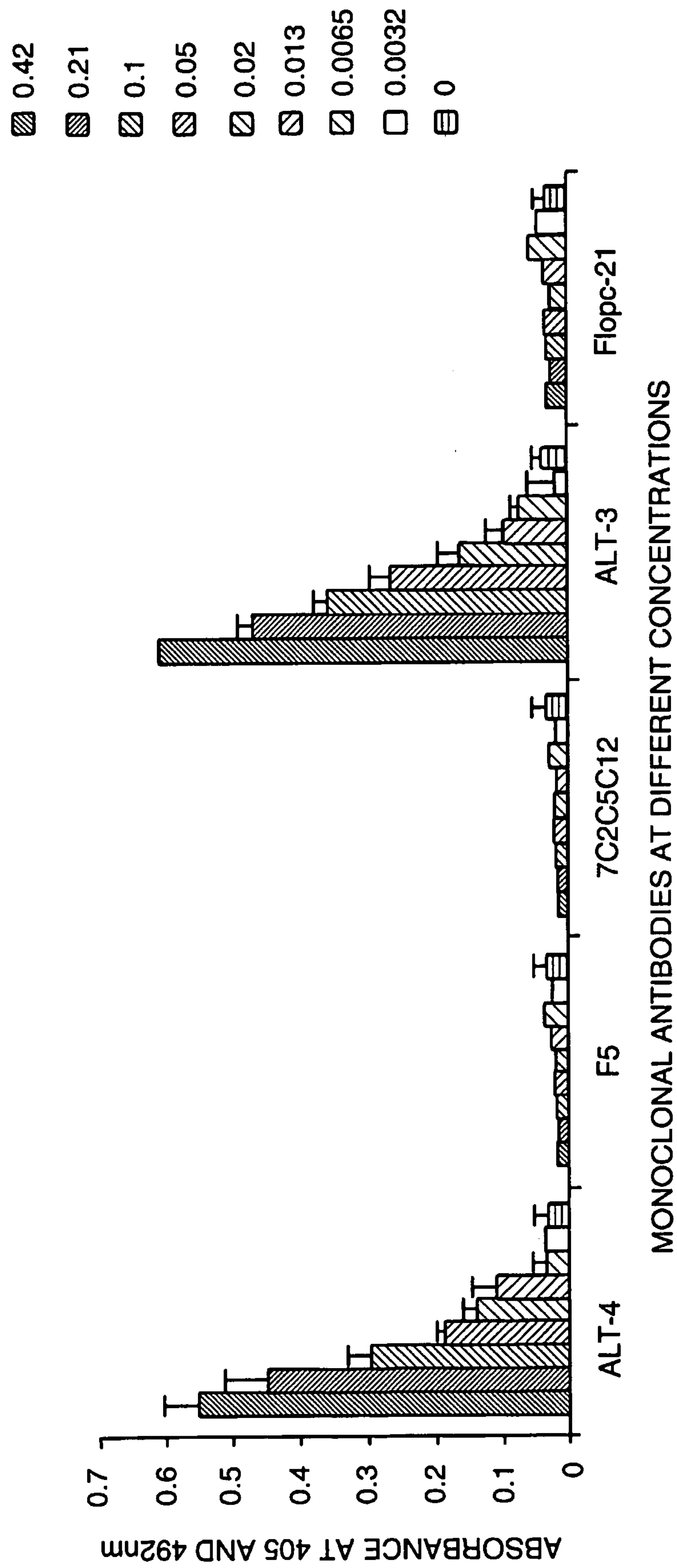

0.42
0.21
0.1
0.05
0.02
0.013
0.0065
0.0032
0
ABSORBANCE AT 405 AND 492nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ALT-4
F5
7C2C5C12
ALT-3
Flopc-21
MONOCLONAL ANTIBODIES AT DIFFERENT CONCENTRATIONS

FIG. 7

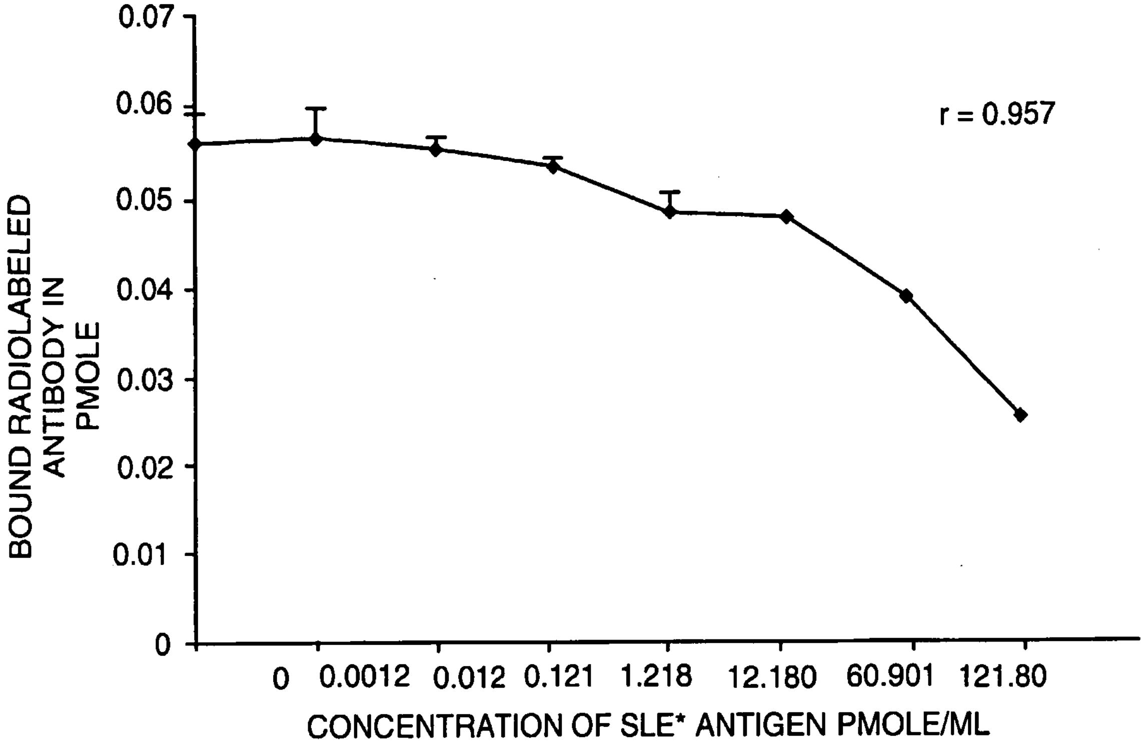

0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
BOUND RADIOLABELED ANTIBODY IN PMOLE
r = 0.957
0 0.0012 0.012 0.121 1.218 12.180 60.901 121.80
CONCENTRATION OF SLE* ANTIGEN PMOLE/ML

FIG. 8

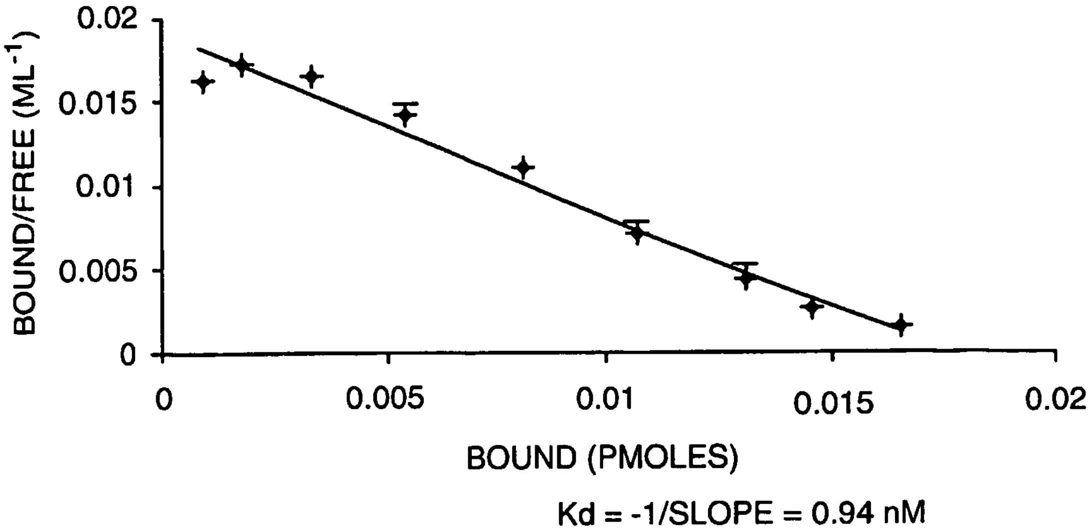

0.02
0.015
0.01
0.005
0
BOUND/FREE (ML-1)
0 0.005 0.01 0.015 0.02
BOUND (PMOLES)
Kd = -1/SLOPE = 0.94 nM

FIG. 9

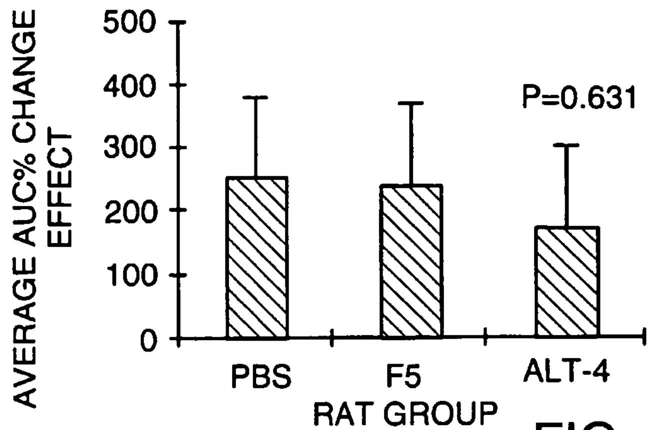


FIG. 10A

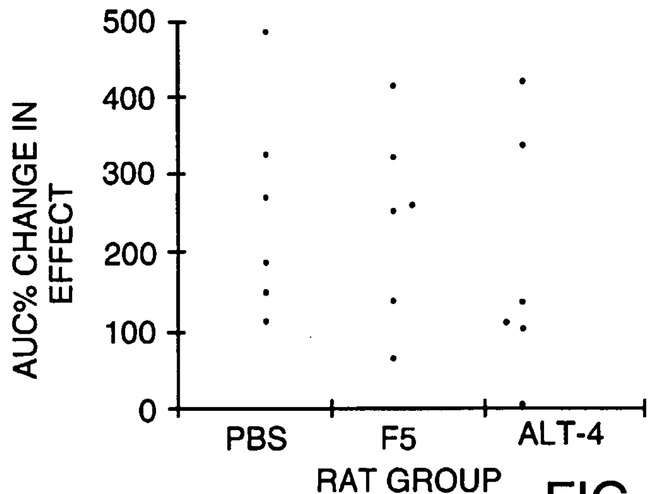


FIG. 10B

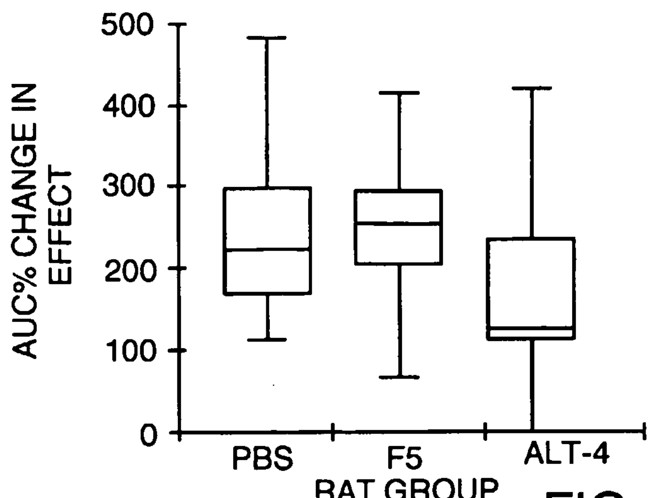


FIG. 10C

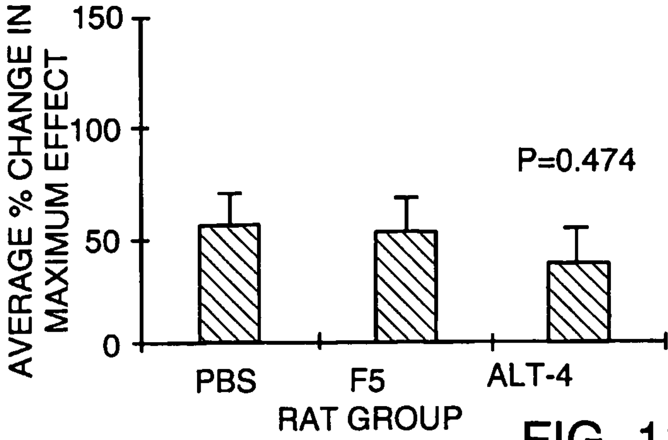


FIG. 11A

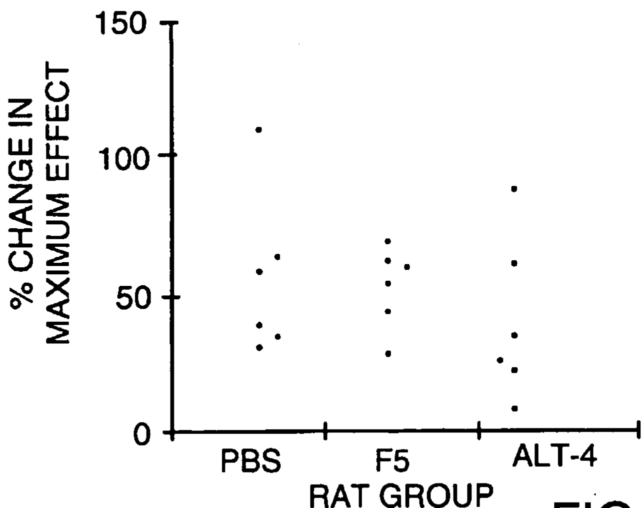


FIG. 11B

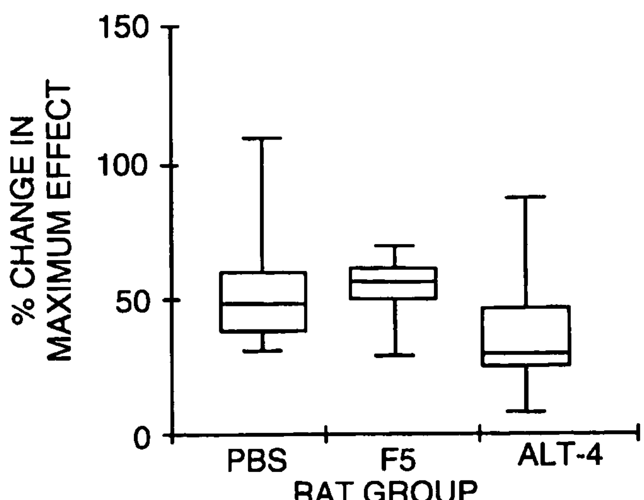


FIG. 11C

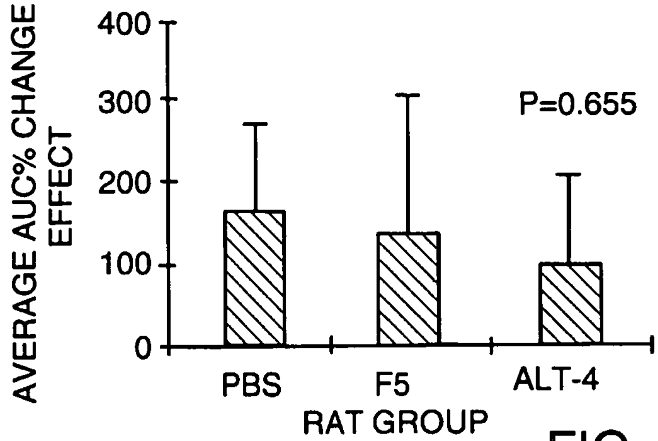


FIG. 12A

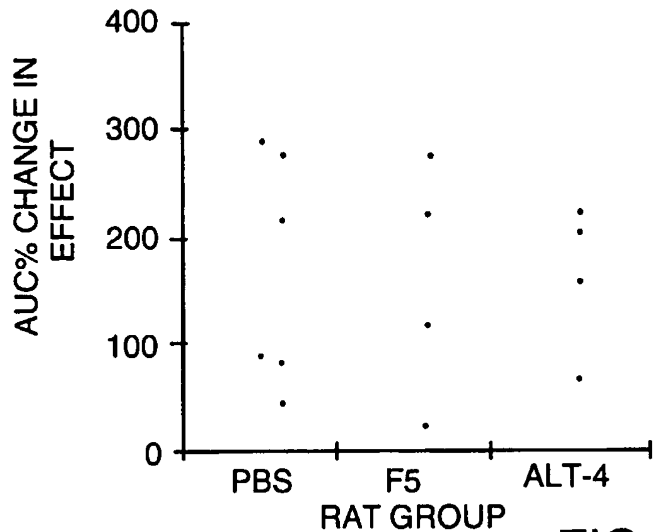


FIG. 12B

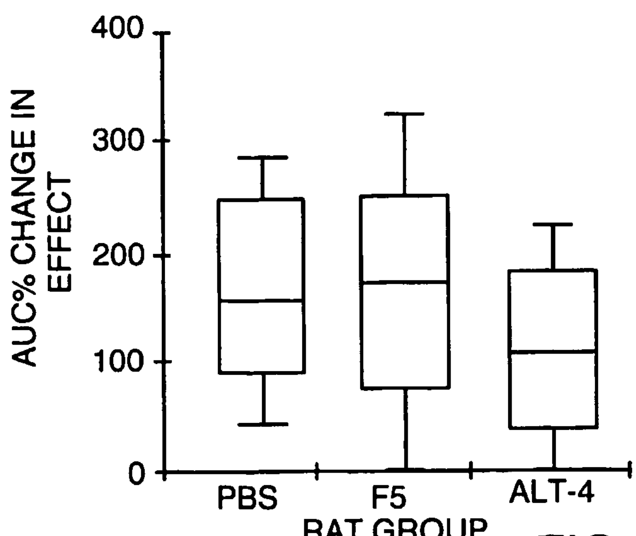


FIG. 12C

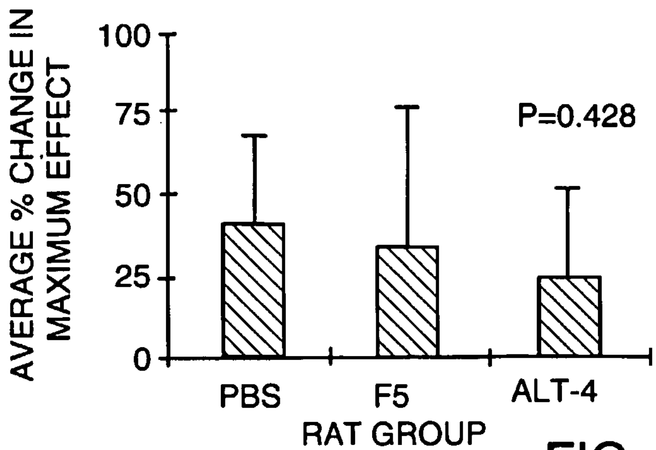


FIG. 13A

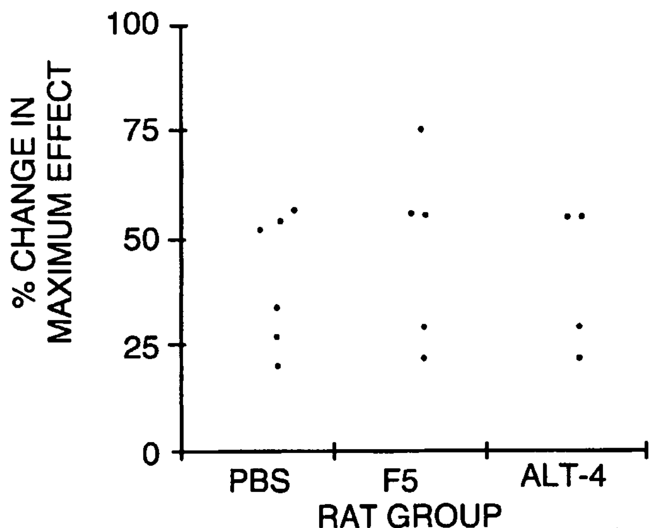


FIG. 13B

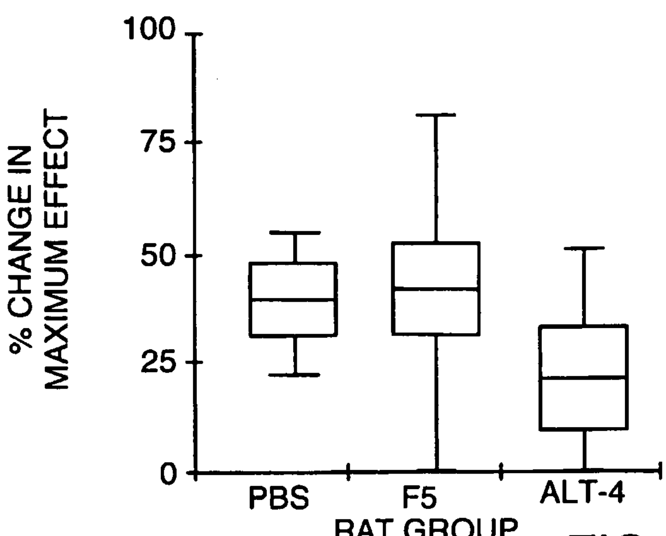


FIG. 13C

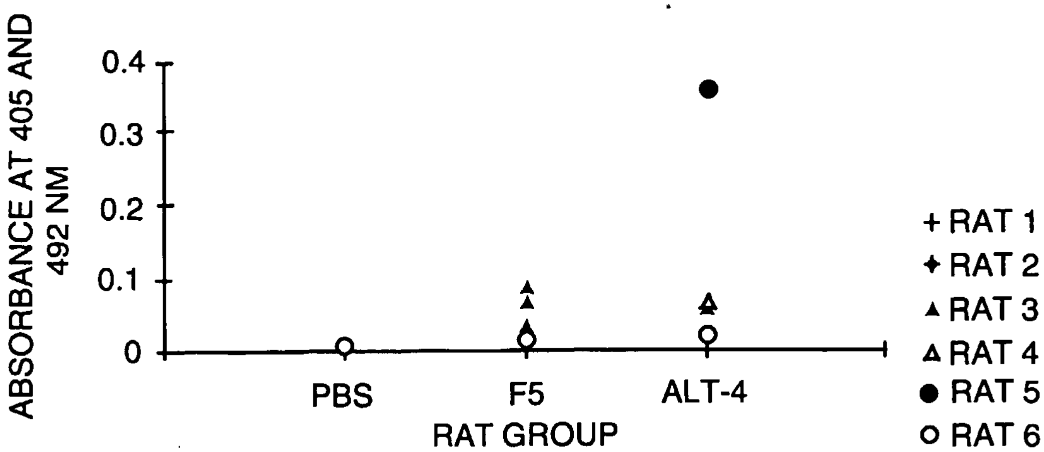


FIG. 14A

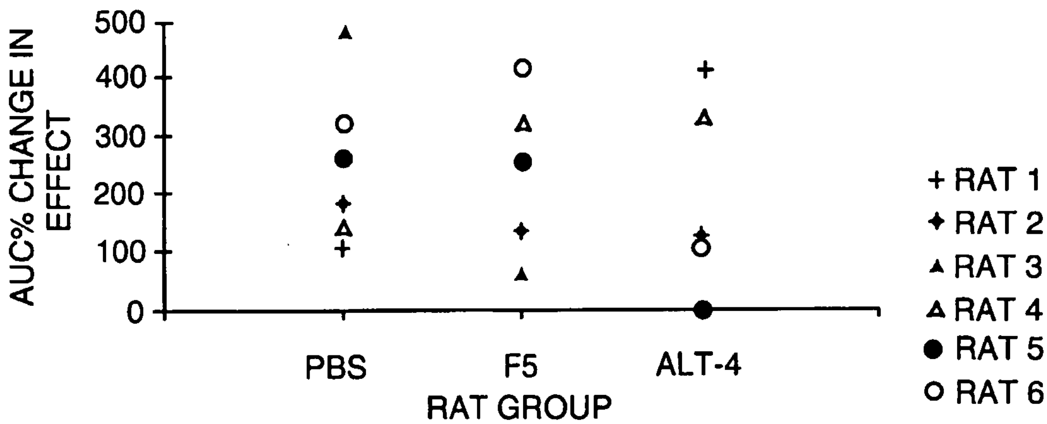


FIG. 14B

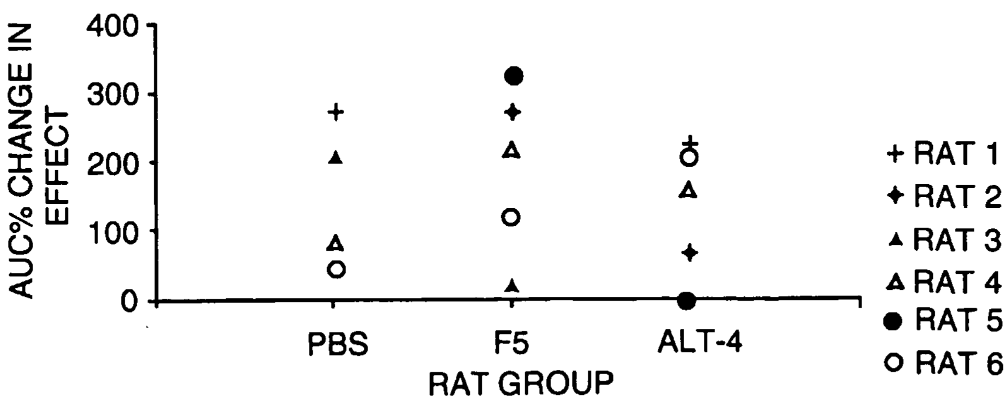


FIG. 14C

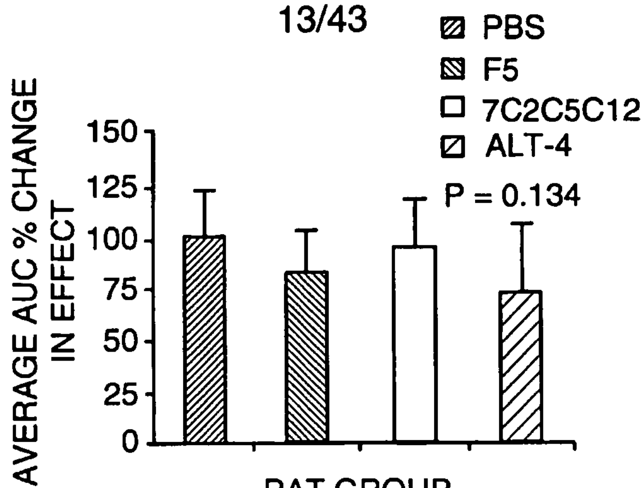


FIG. 15A

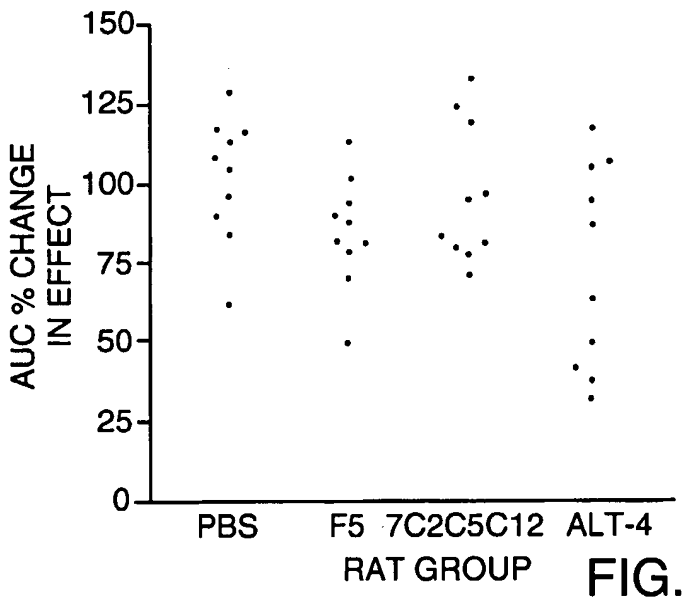


FIG. 15B

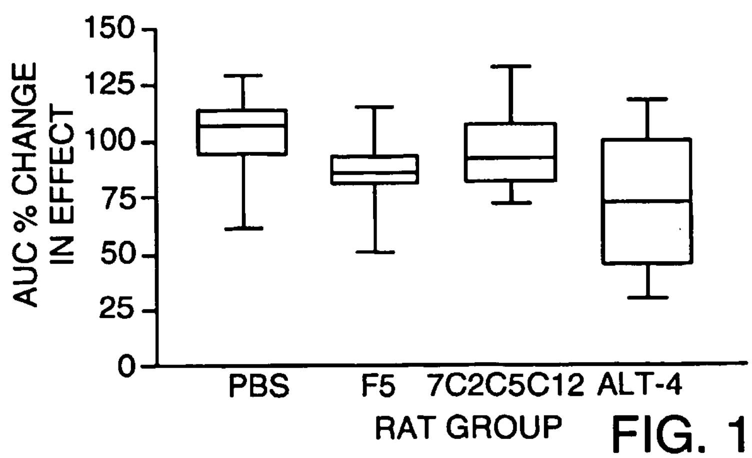


FIG. 15C

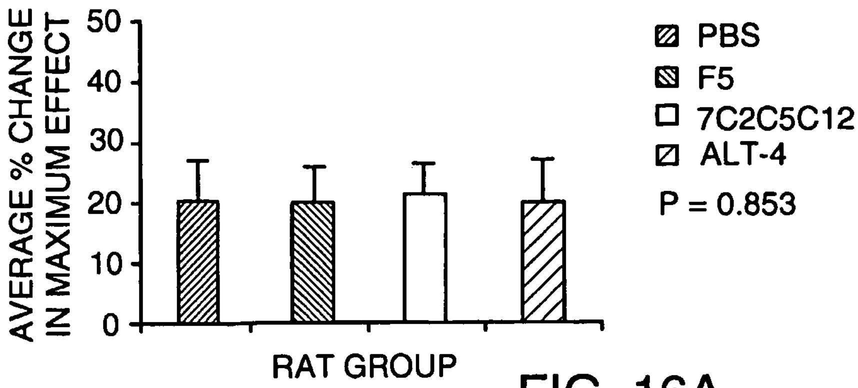


FIG. 16A

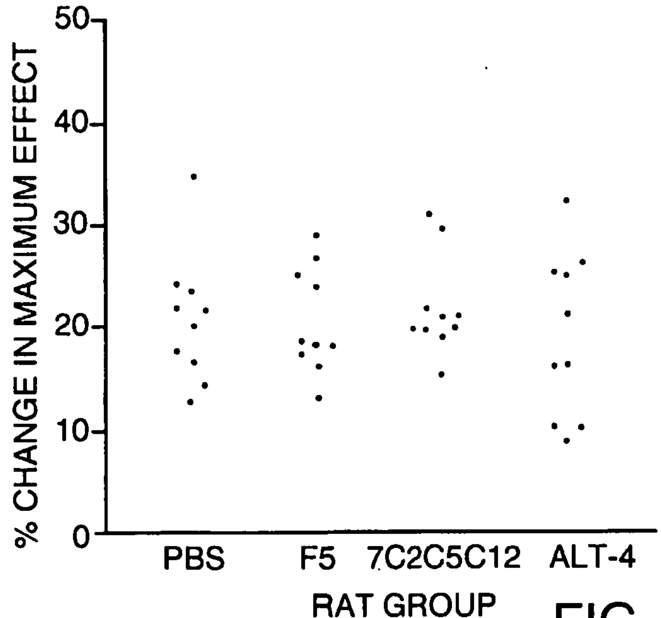


FIG. 16B

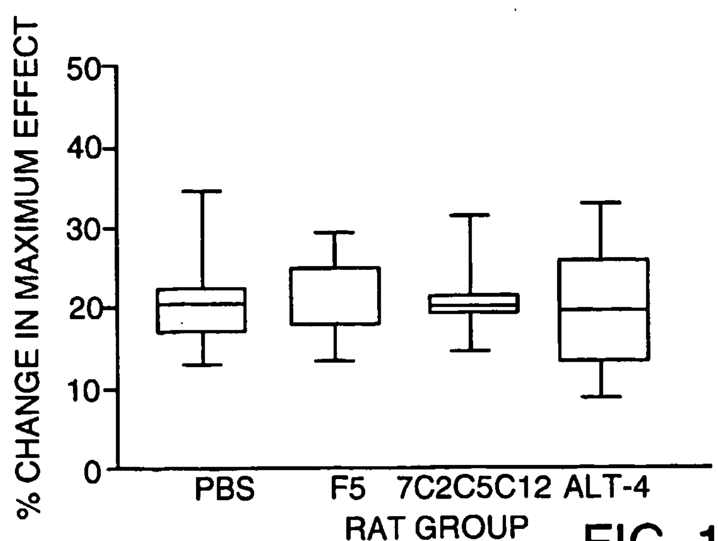


FIG. 16C

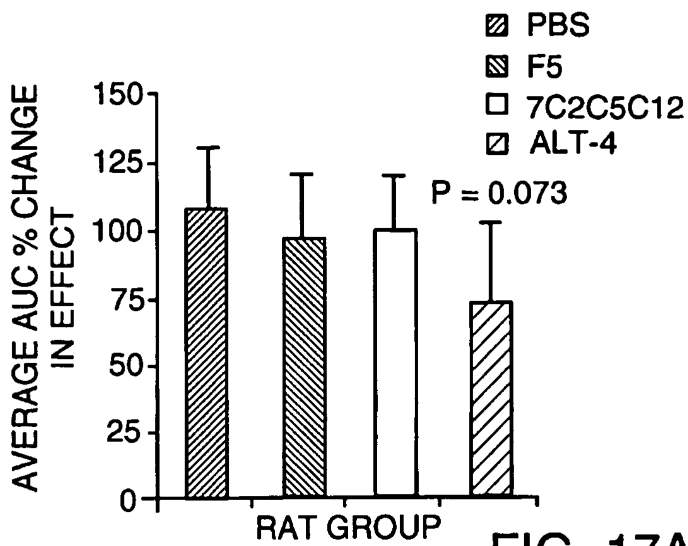


FIG. 17A

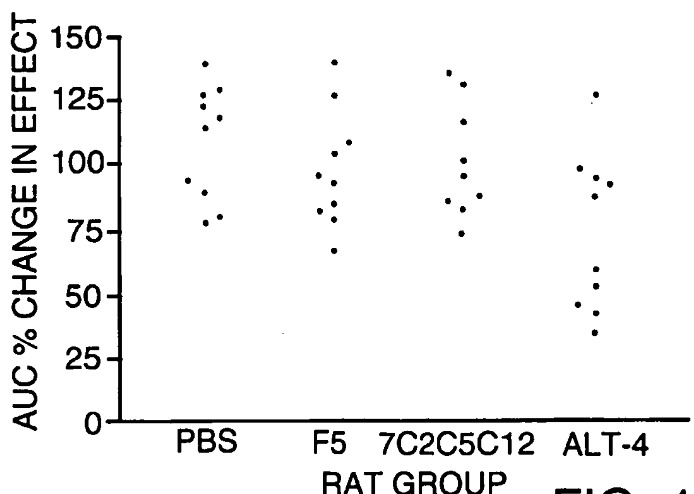


FIG. 17B

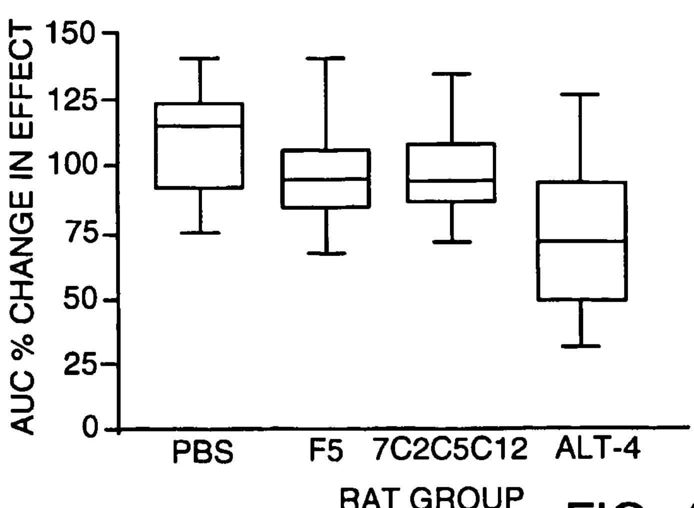


FIG. 17C

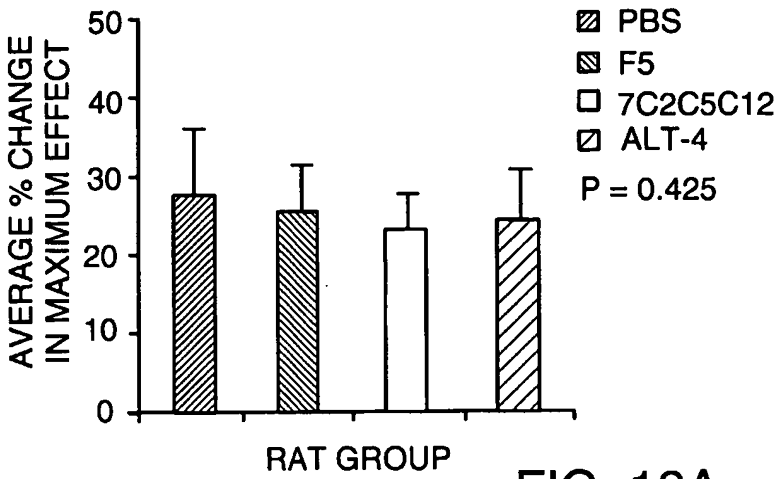


FIG. 18A

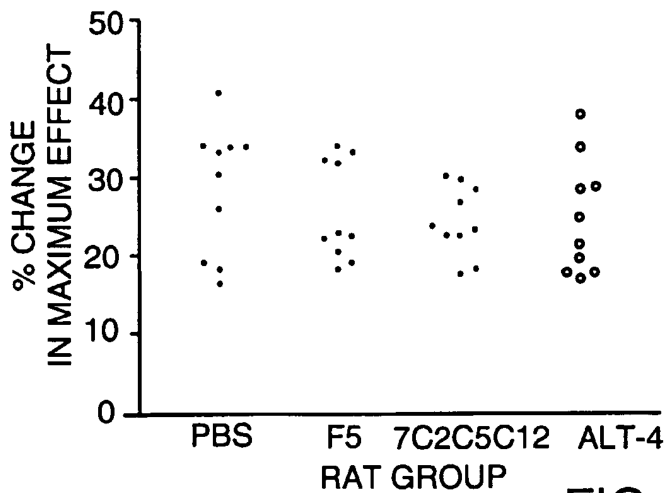


FIG. 18B

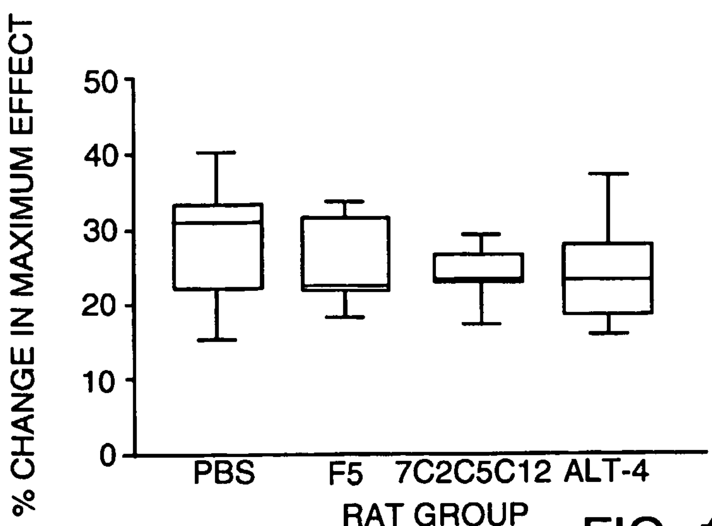


FIG. 18C

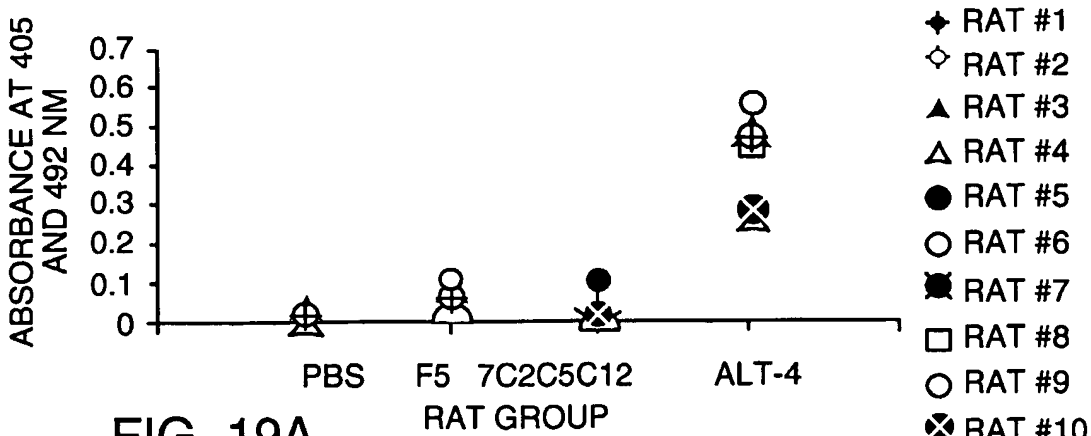


FIG. 19A

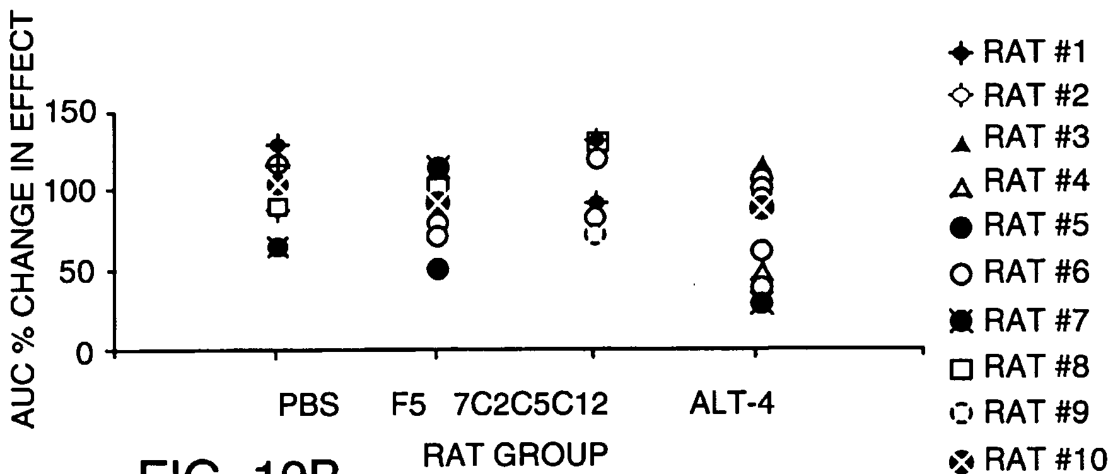


FIG. 19B

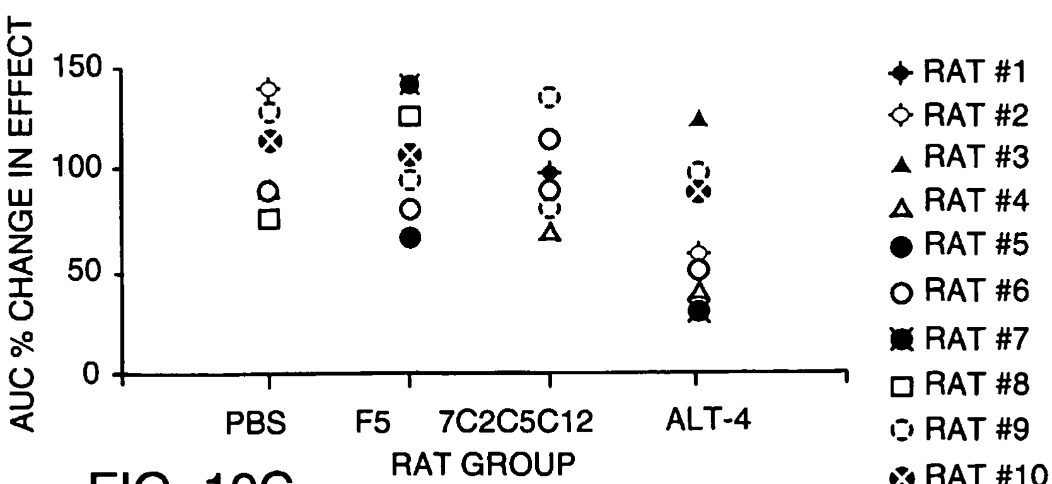


FIG. 19C

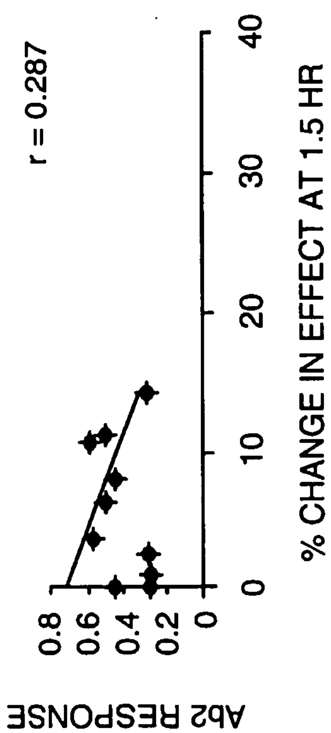


FIG. 20A

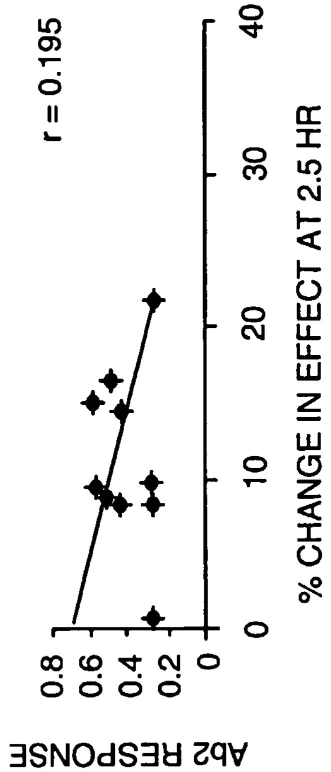


FIG. 20B

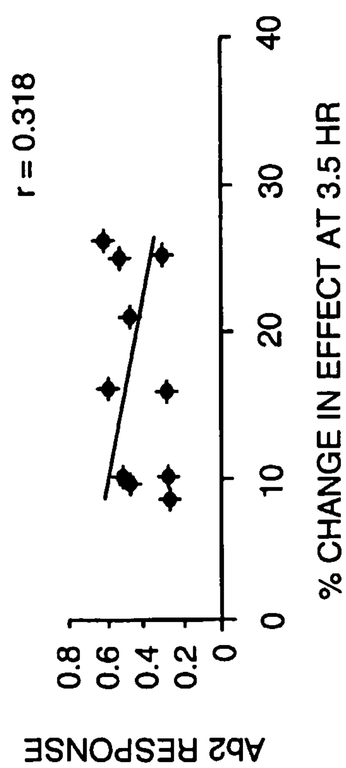


FIG. 20C

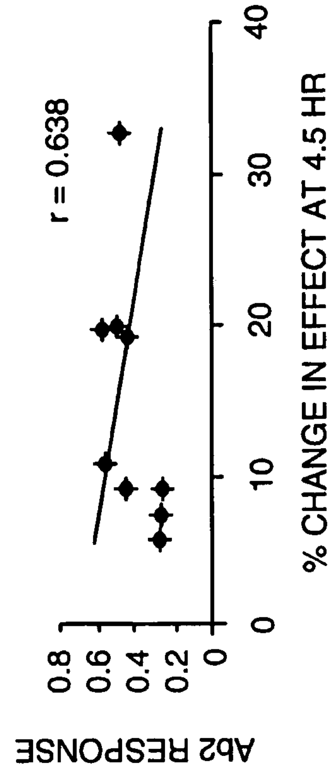


FIG. 20D

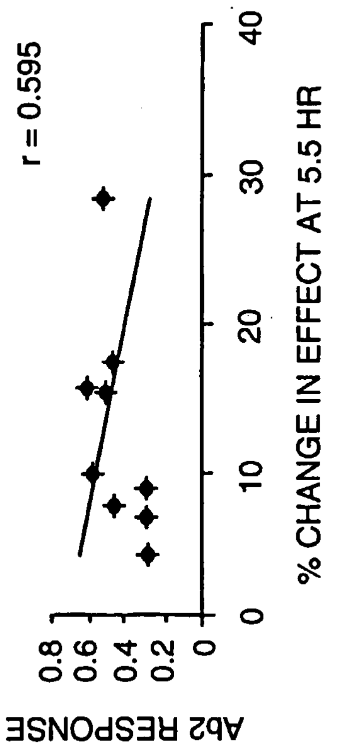


FIG. 20E

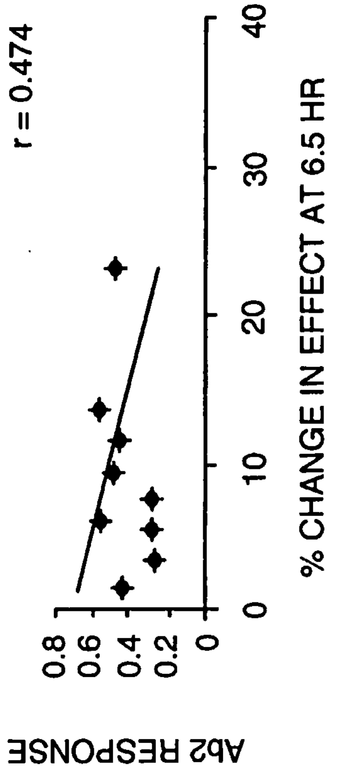


FIG. 20F

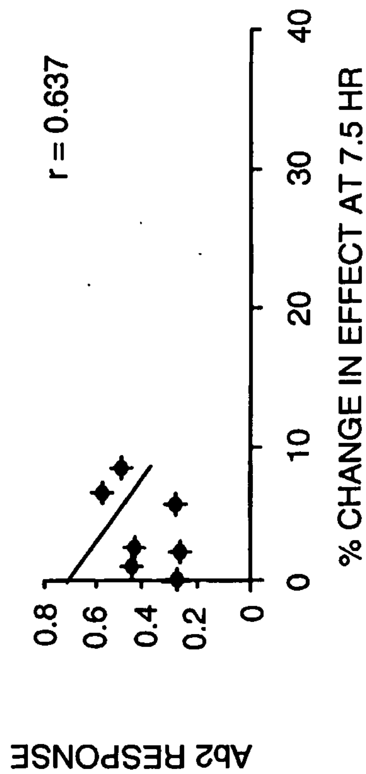


FIG. 20G

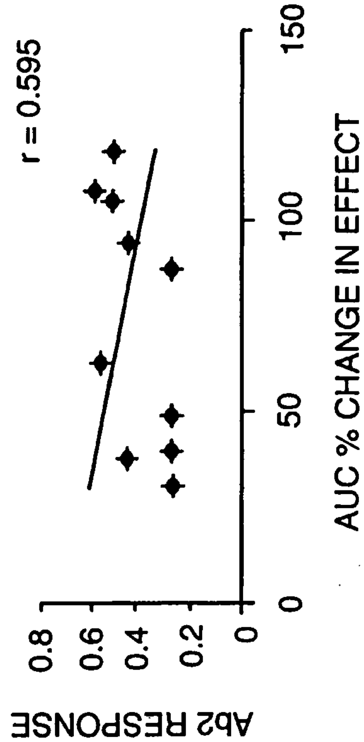


FIG. 20H

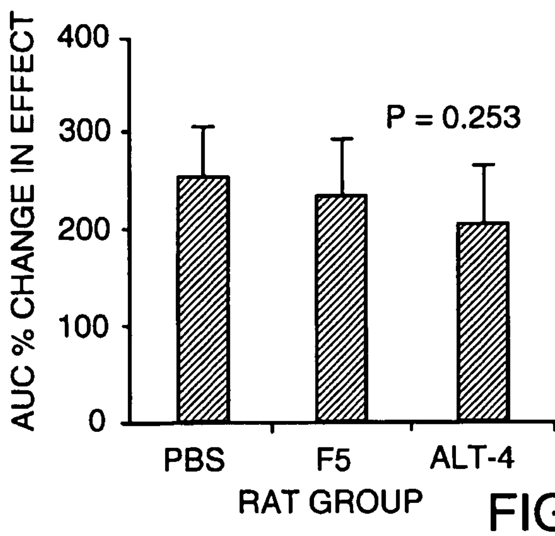


FIG. 21A

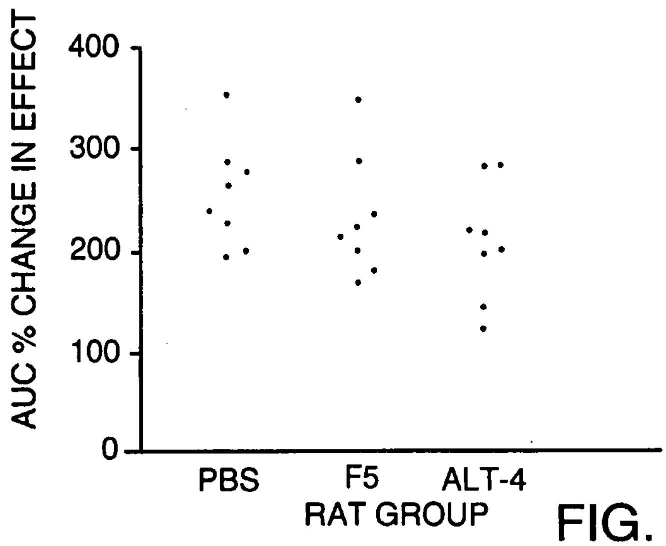


FIG. 21B

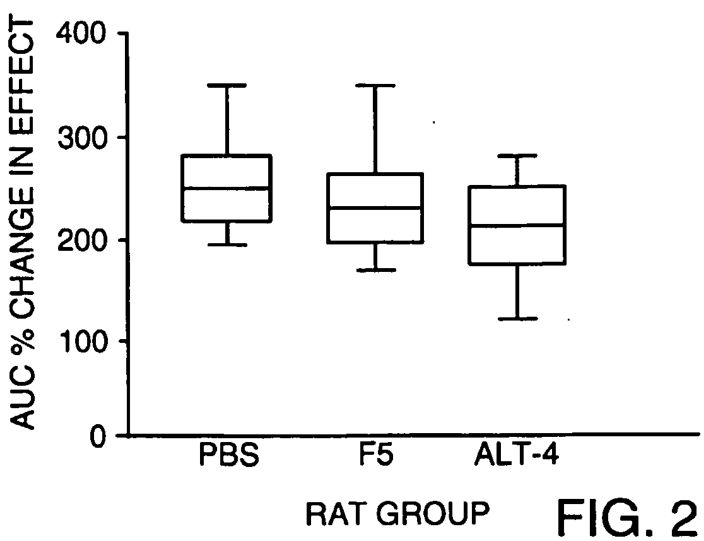


FIG. 21C

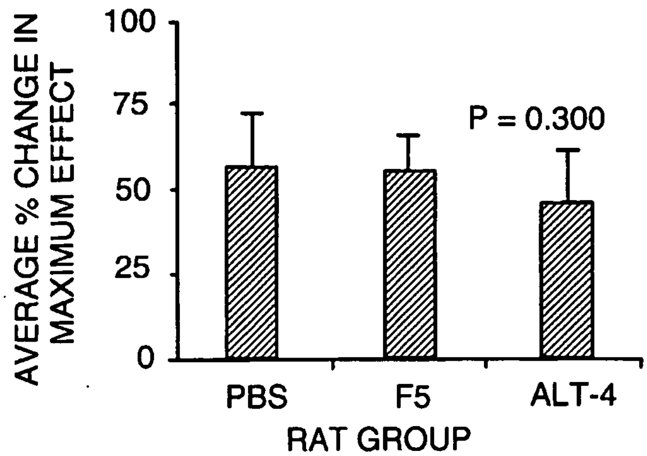


FIG. 22A

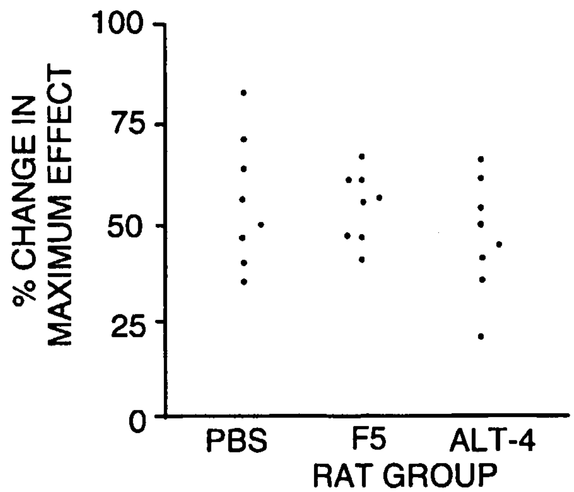


FIG. 22B

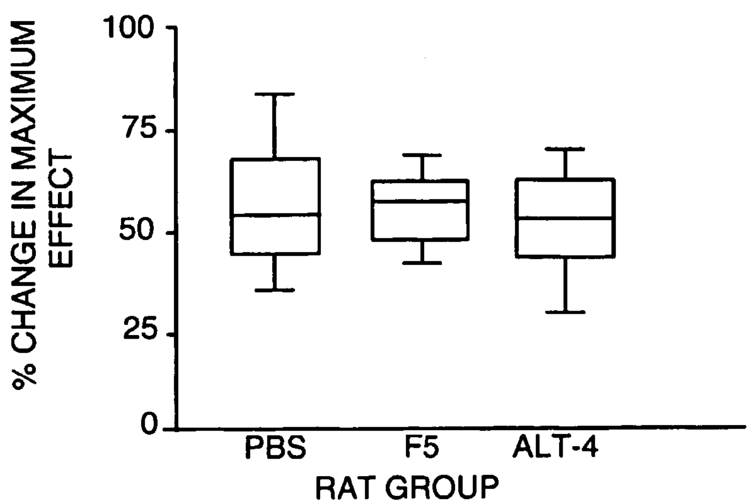


FIG. 22C

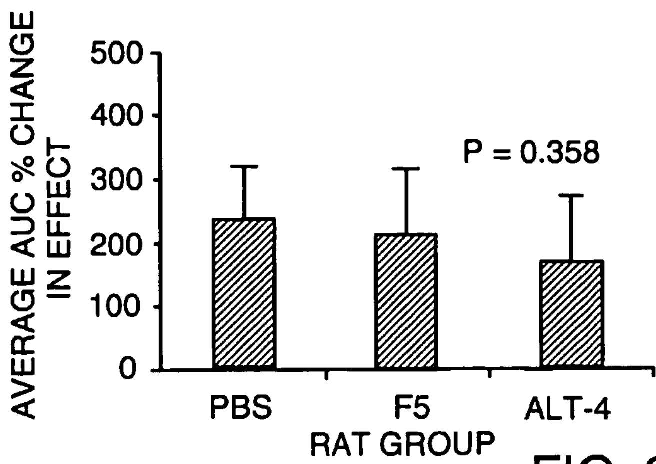


FIG. 23A

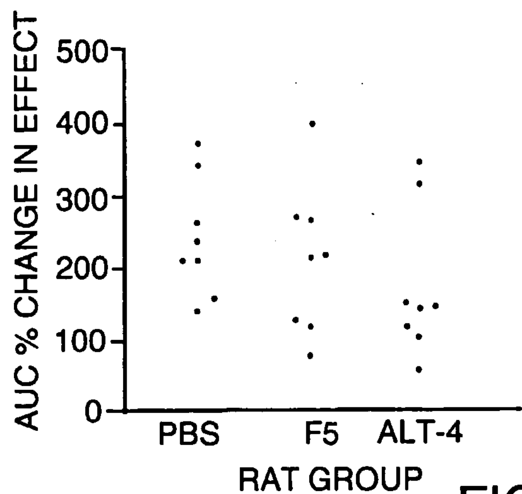


FIG. 23B

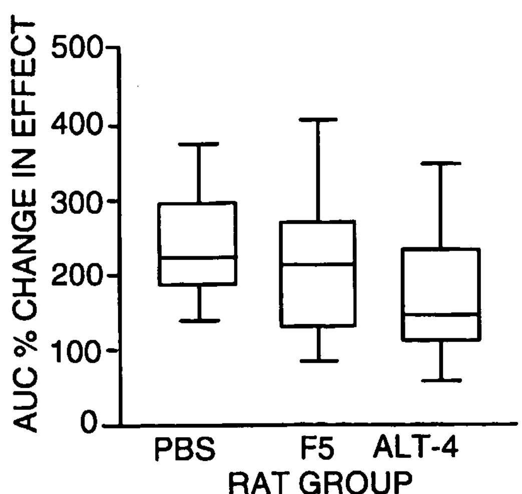


FIG. 23C

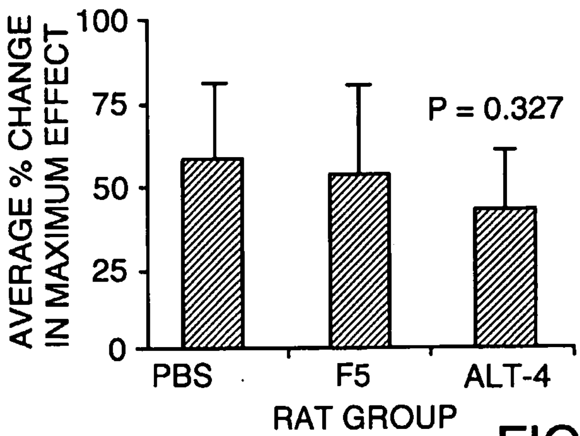


FIG. 24A

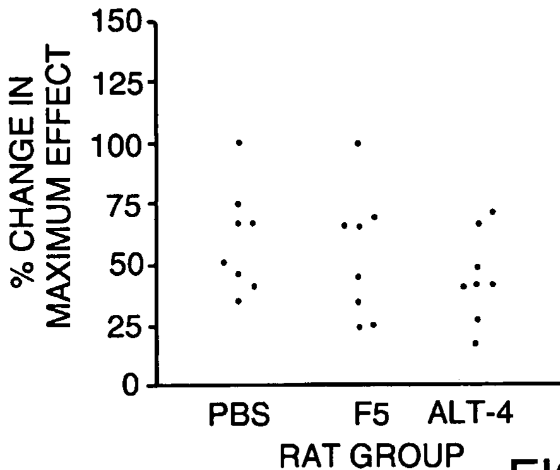


FIG. 24B

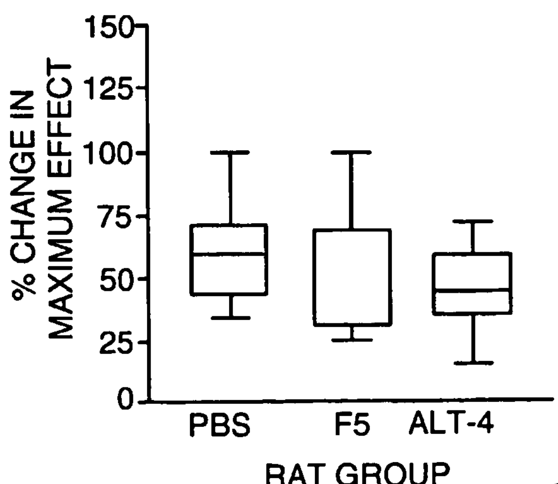


FIG. 24C

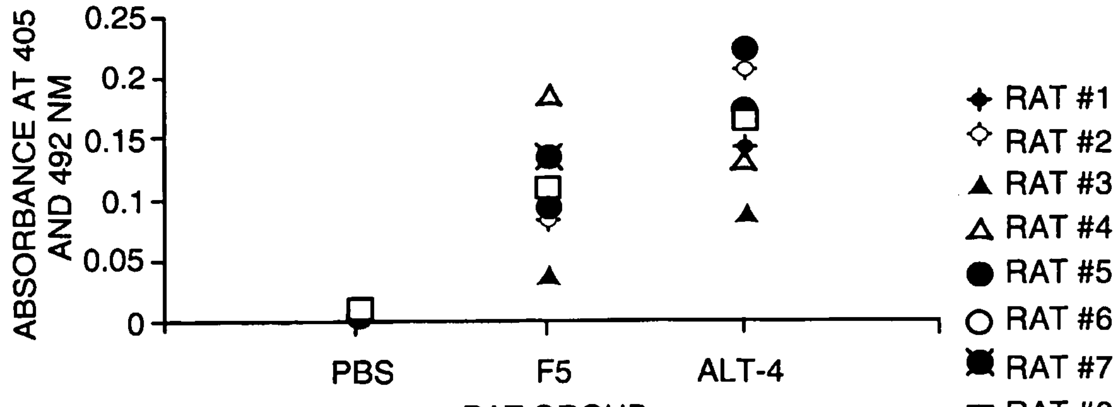


FIG. 25A

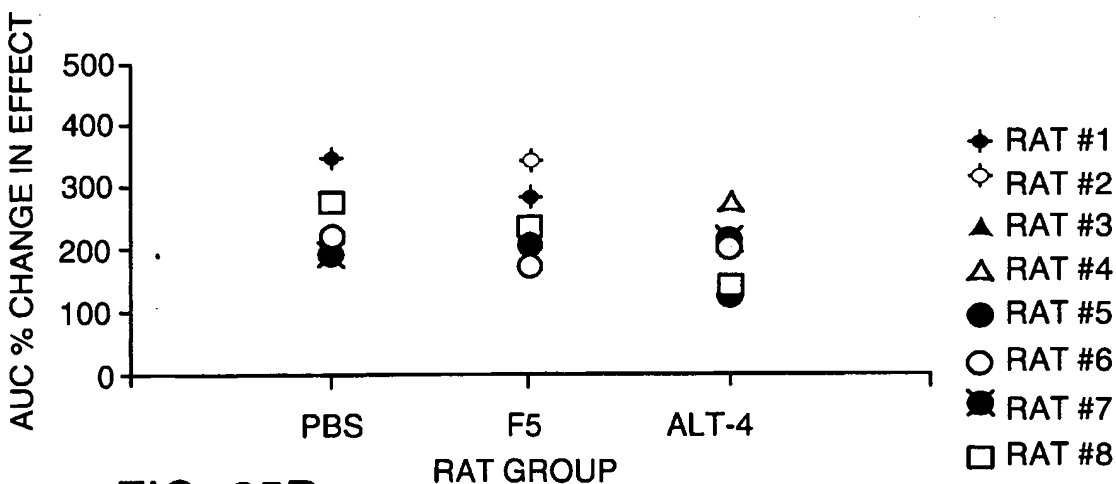


FIG. 25B

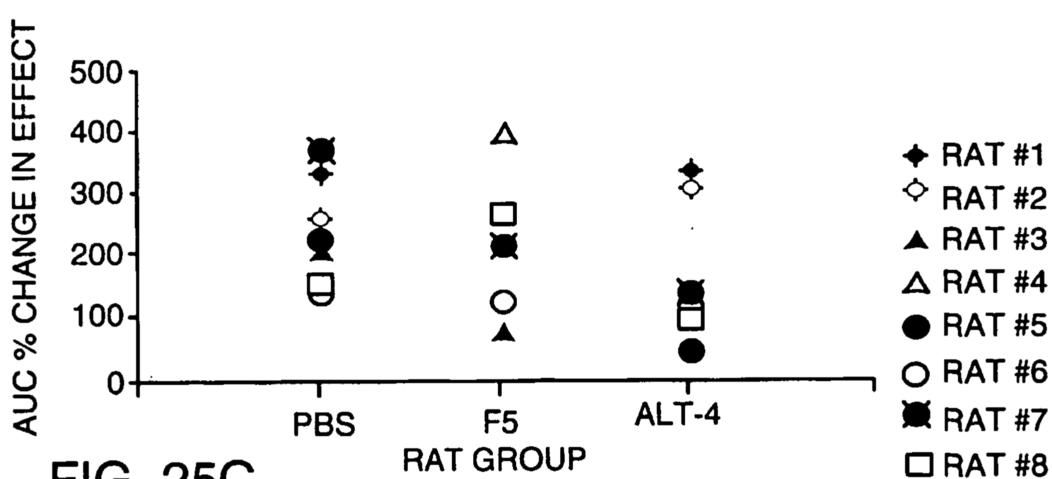


FIG. 25C

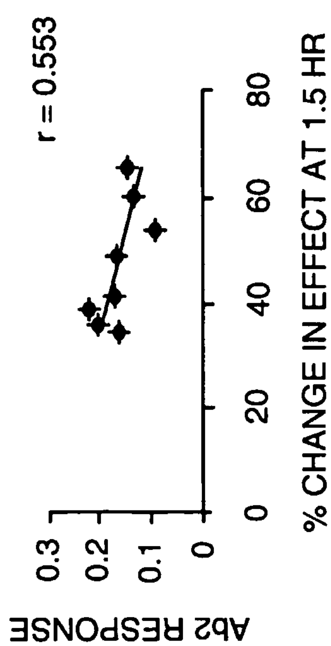


FIG. 26A

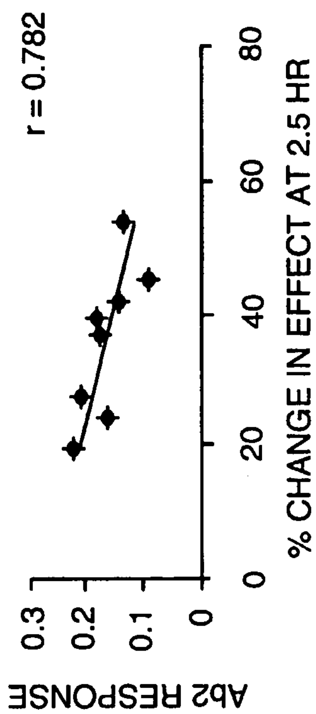


FIG. 26B

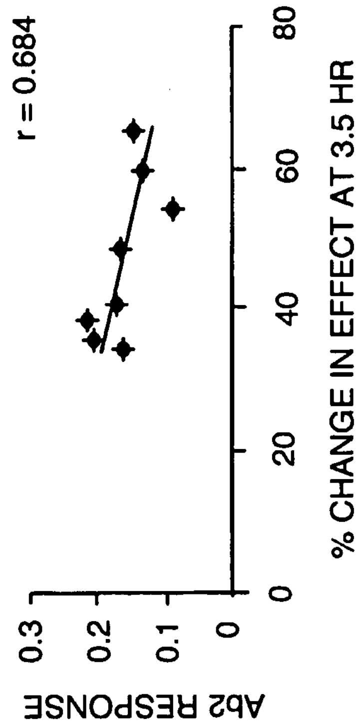


FIG. 26C

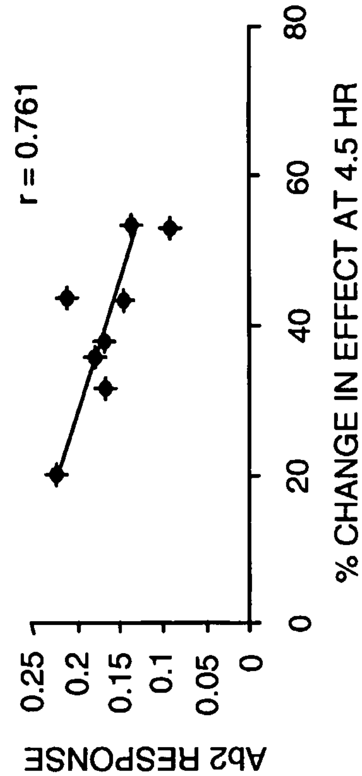


FIG. 26D

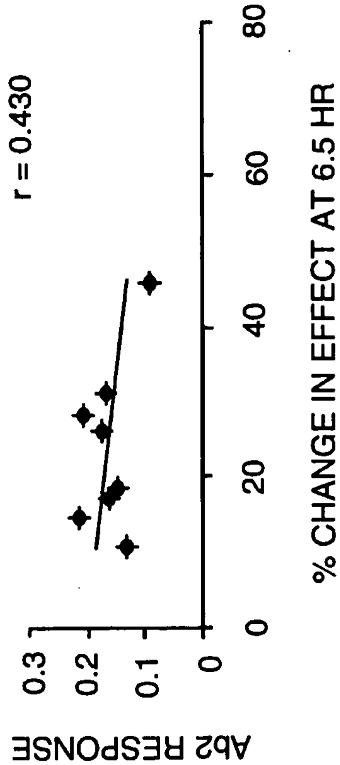


FIG. 26F

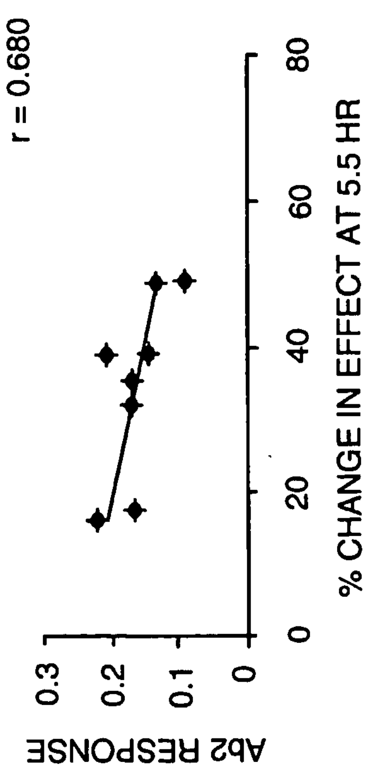


FIG. 26E

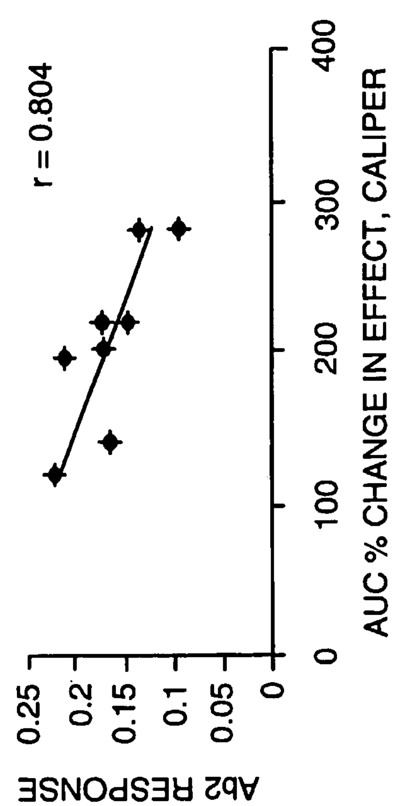


FIG. 26G

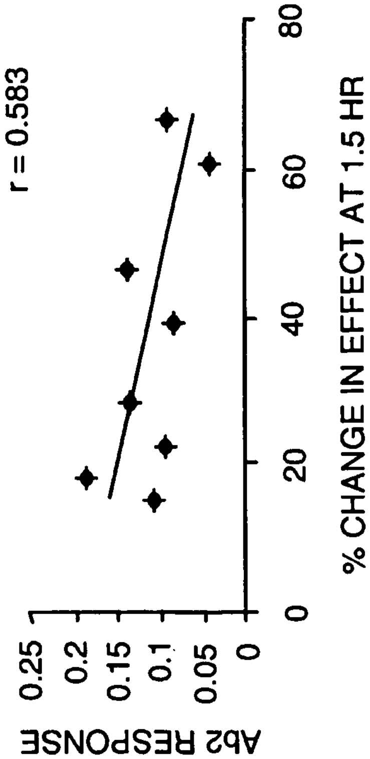


FIG. 27A

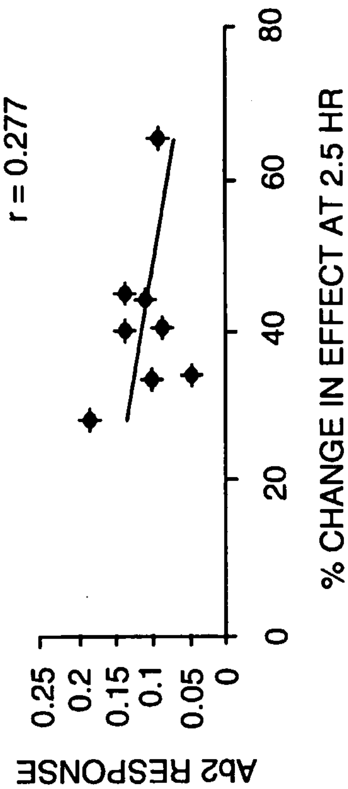


FIG. 27B

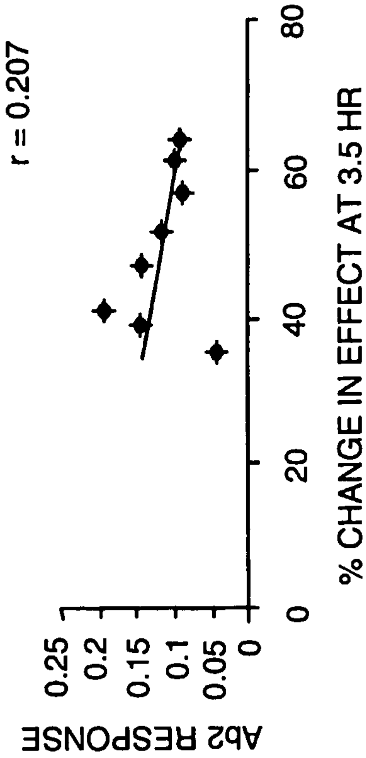


FIG. 27C

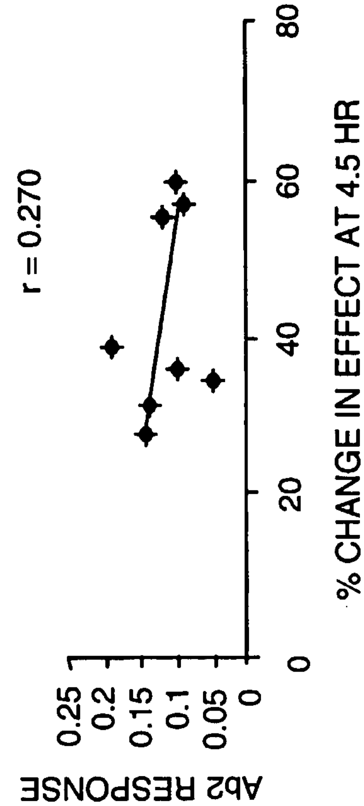


FIG. 27D

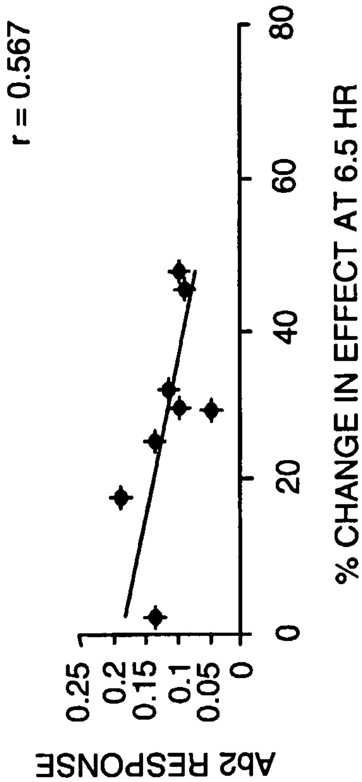


FIG. 27F

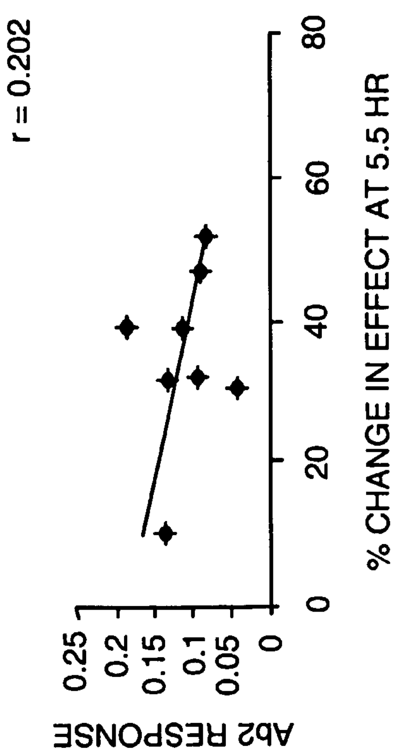


FIG. 27E

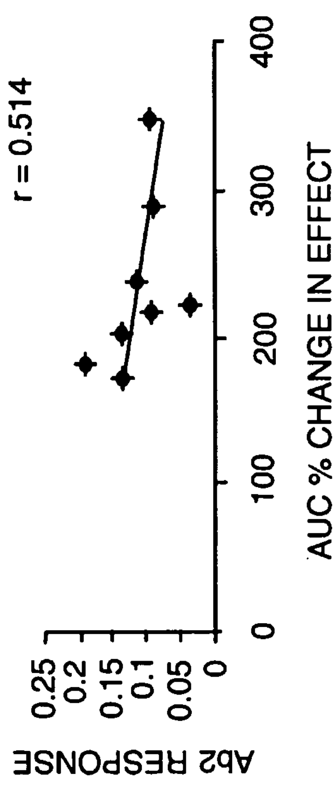


FIG. 27G

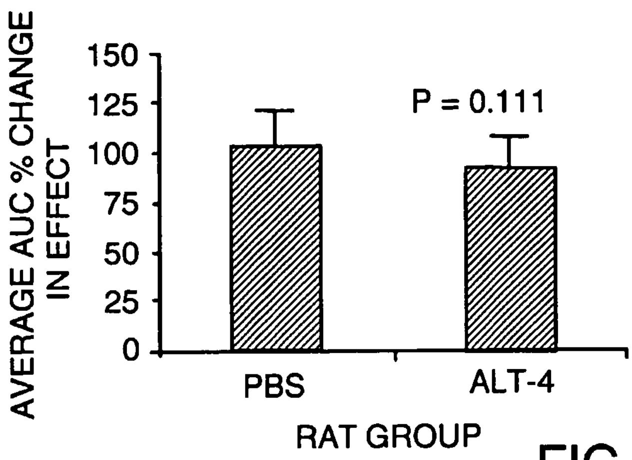


FIG. 28A

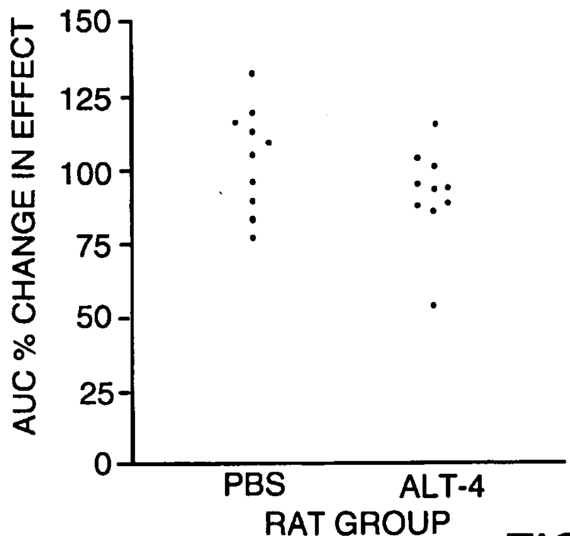


FIG. 28B

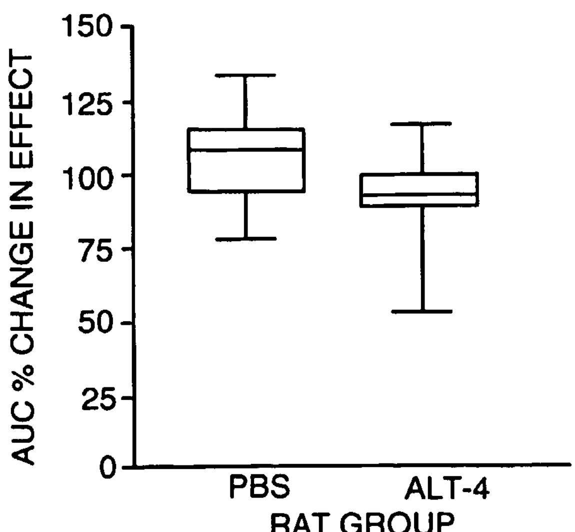


FIG. 28C

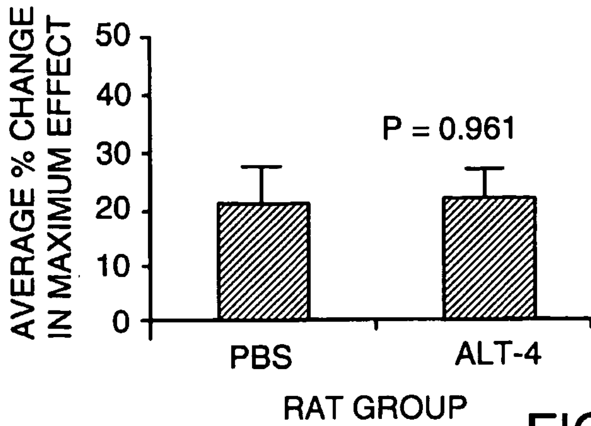


FIG. 29A

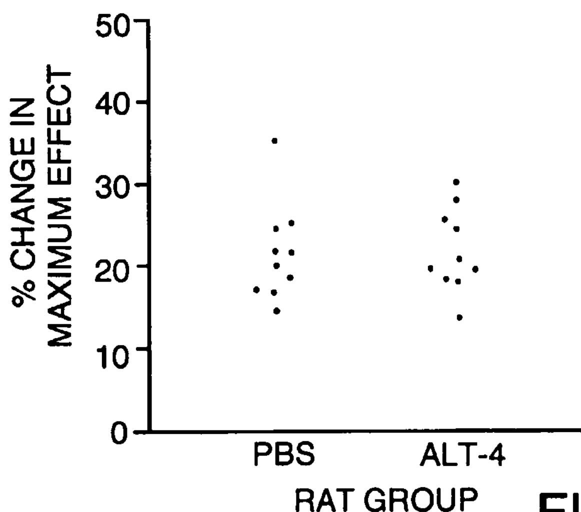


FIG. 29B

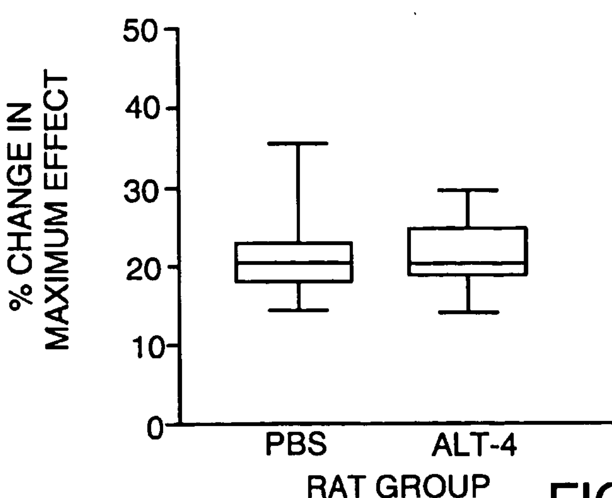


FIG. 29C

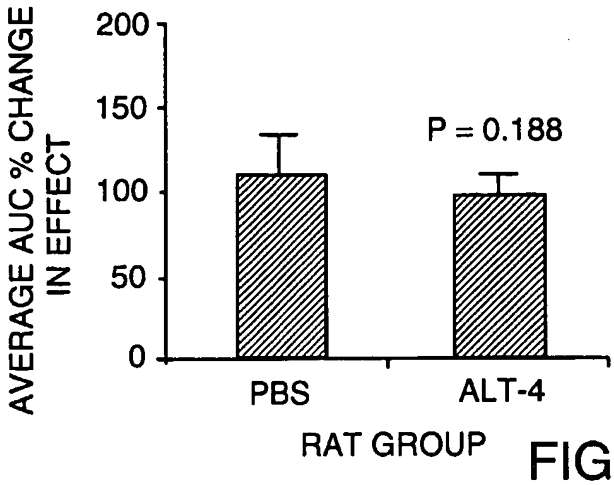


FIG. 30A

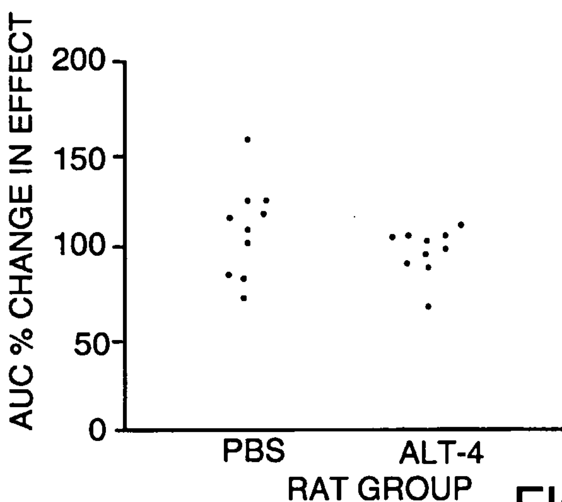


FIG. 30B

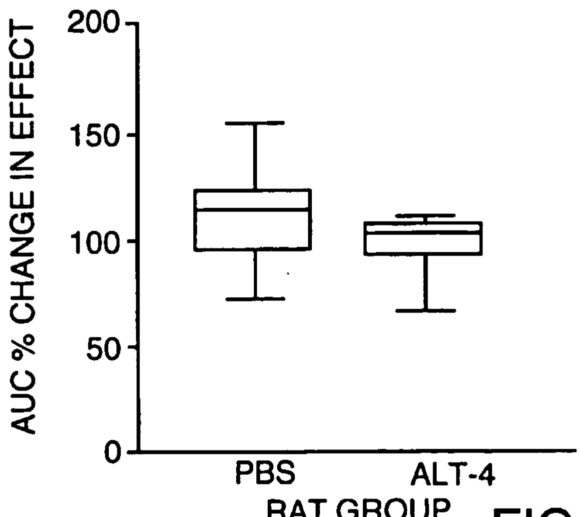


FIG. 30C

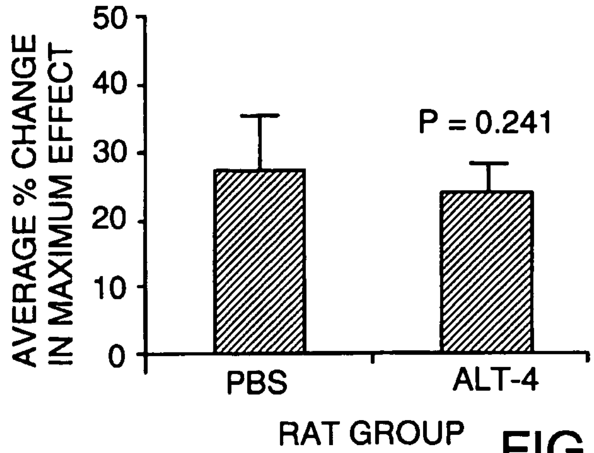


FIG. 31A

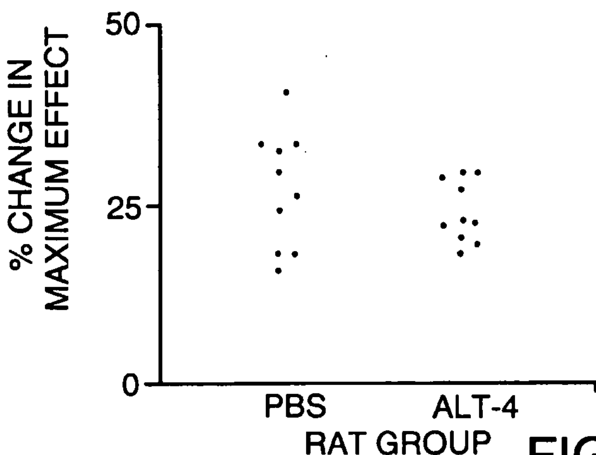


FIG. 31B

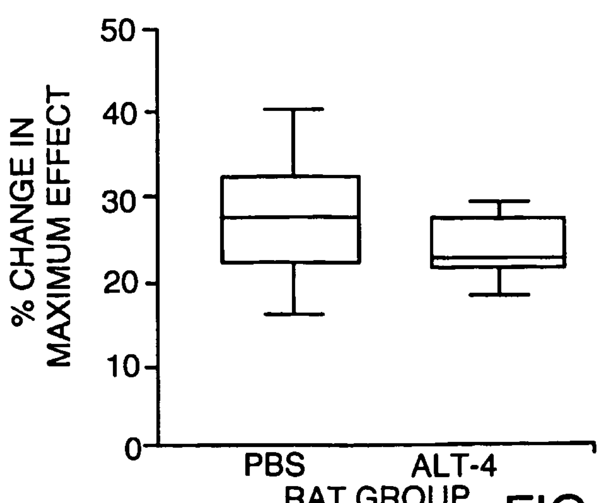


FIG. 31C

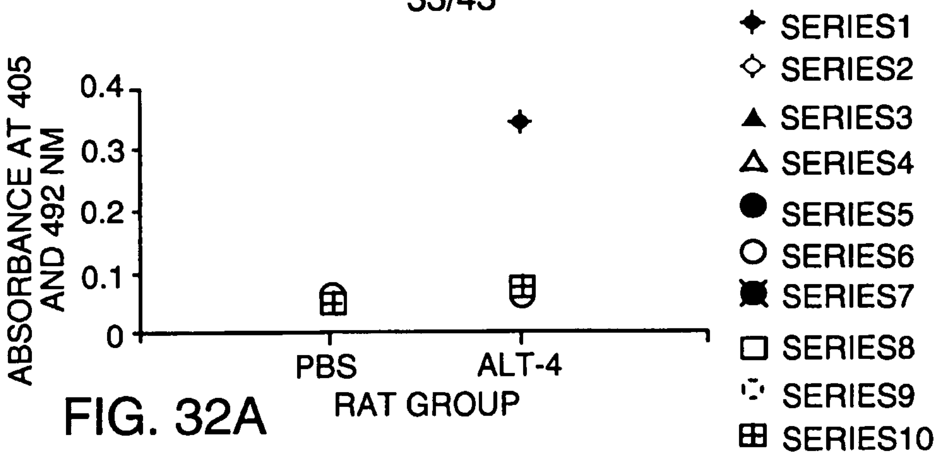


FIG. 32A

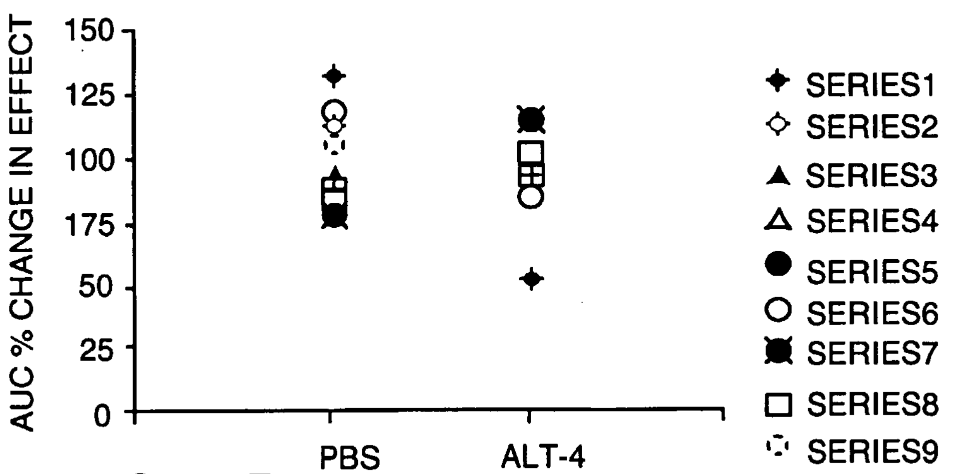


FIG. 32B

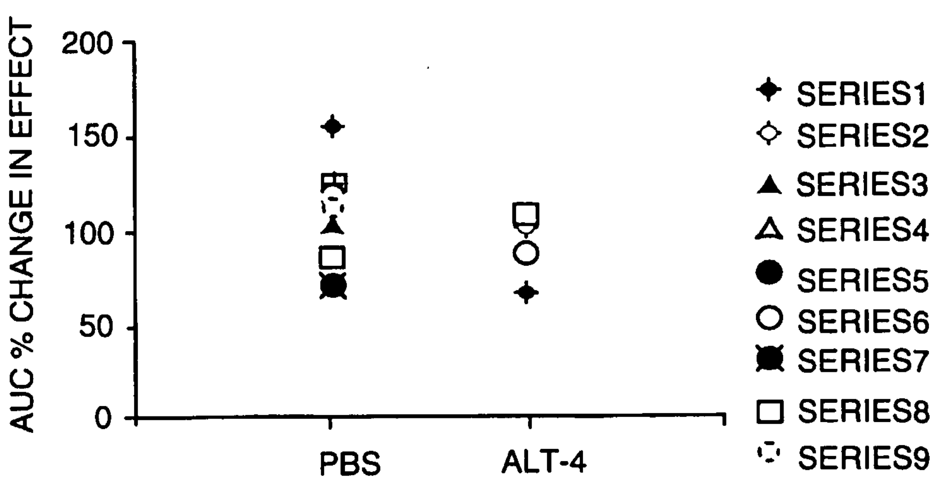


FIG. 32C

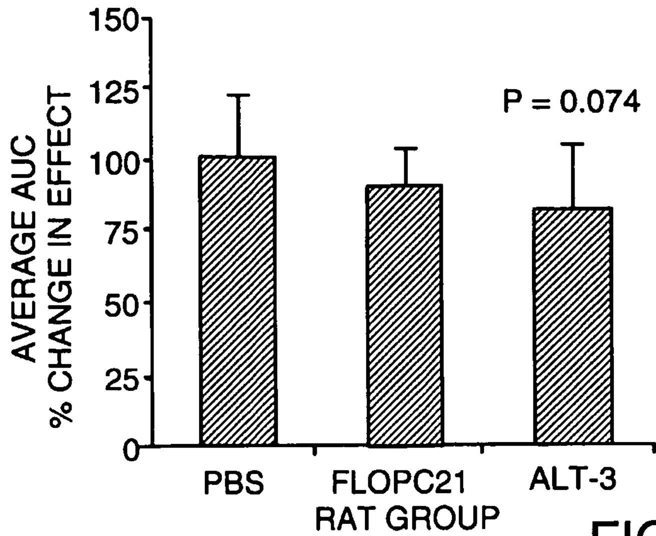


FIG. 33A

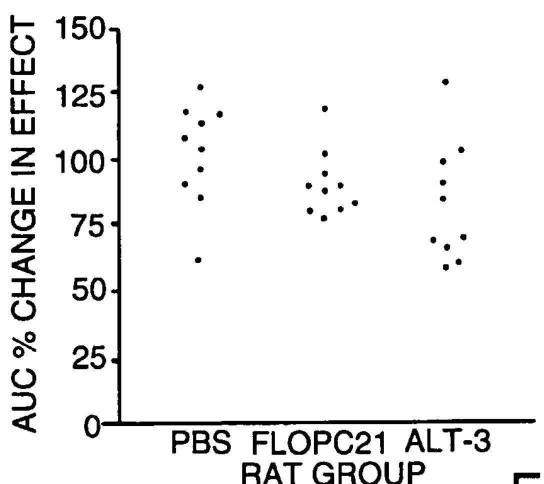


FIG. 33B

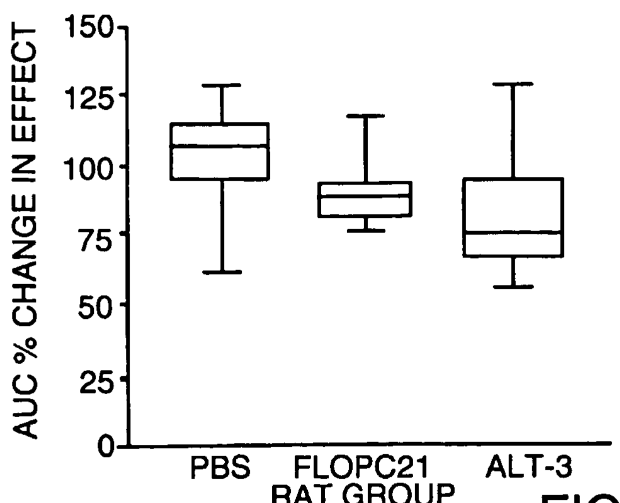


FIG. 33C

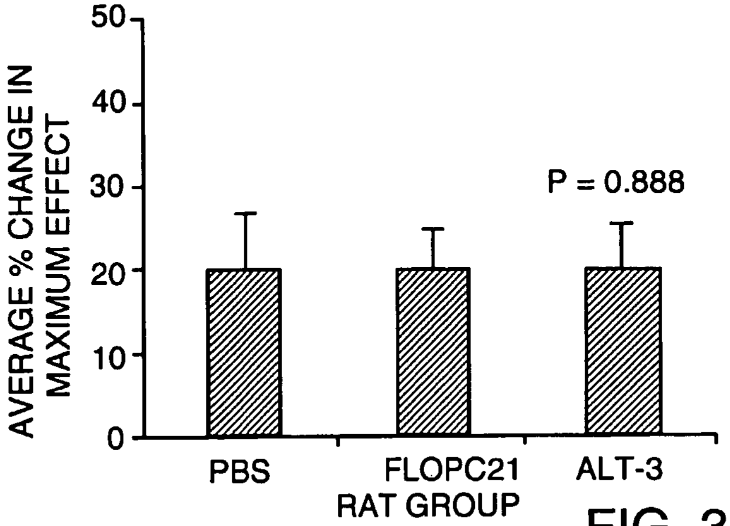


FIG. 34A

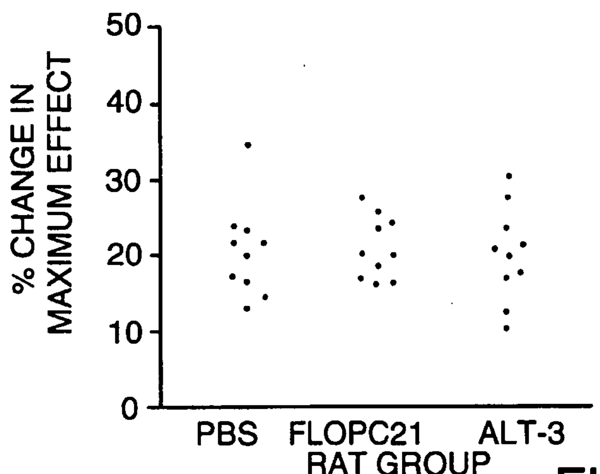


FIG. 34B

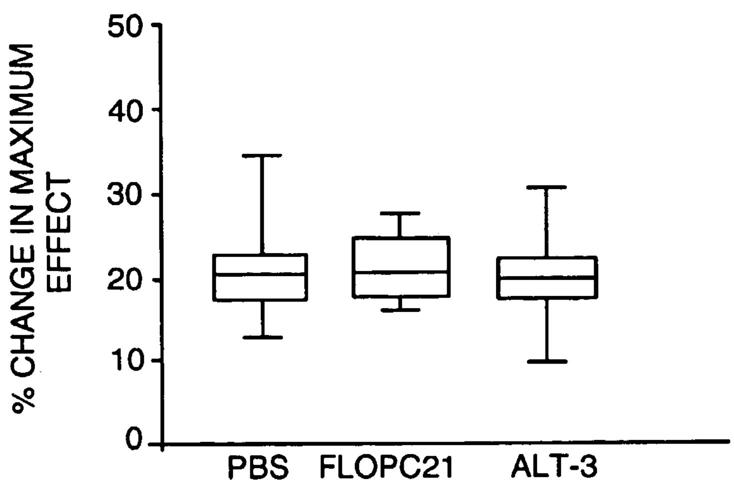


FIG. 34C

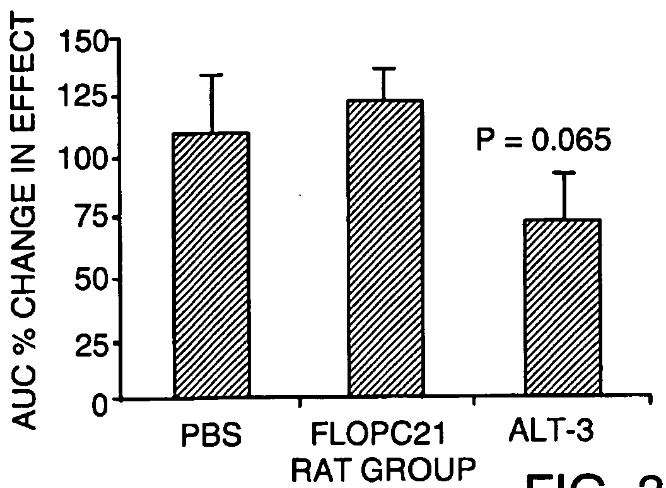


FIG. 35A

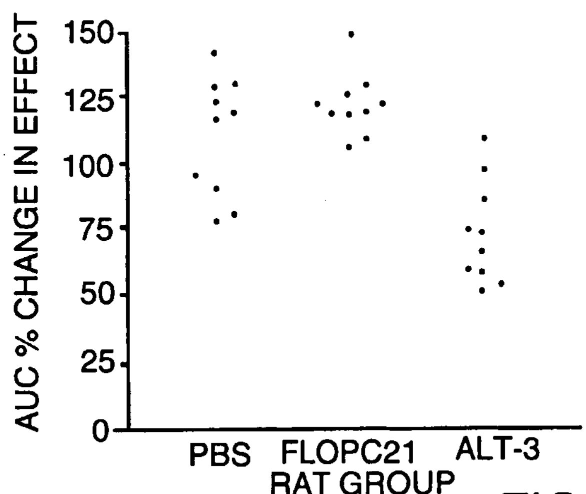


FIG. 35B

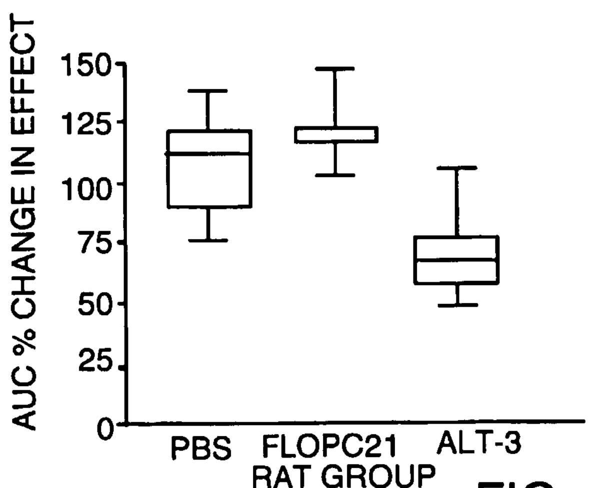


FIG. 35C

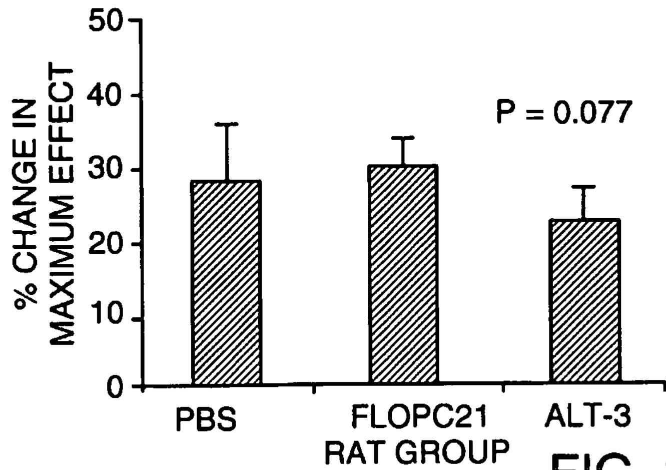


FIG. 36A

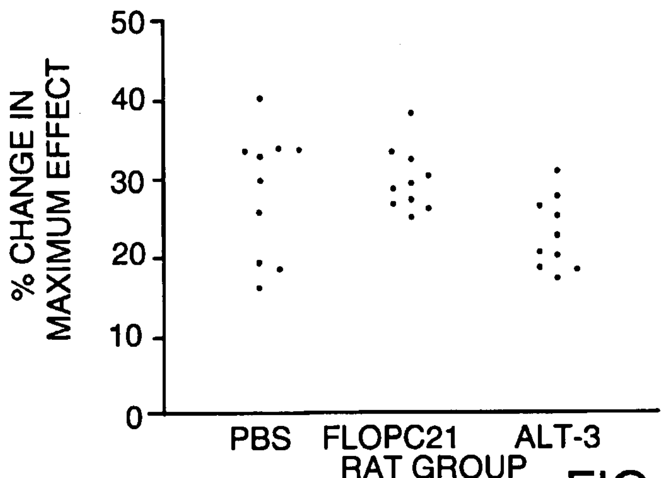


FIG. 36B

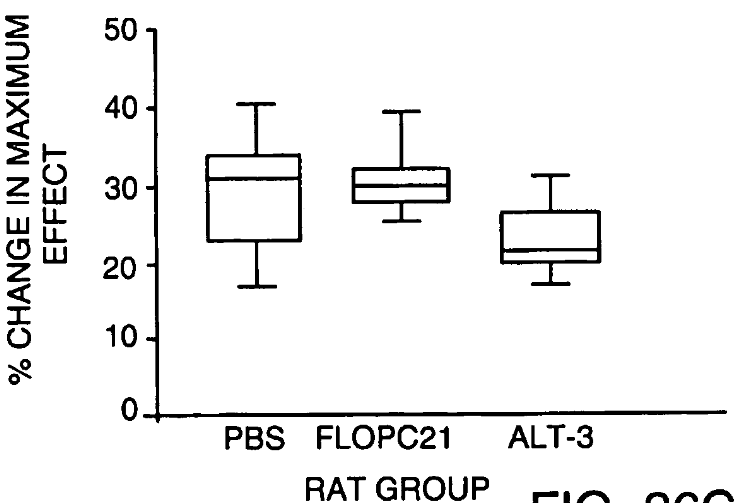


FIG. 36C

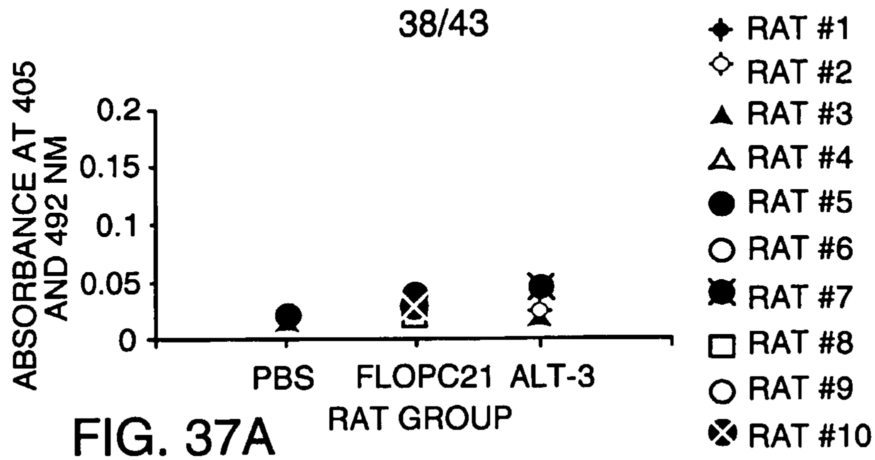


FIG. 37A

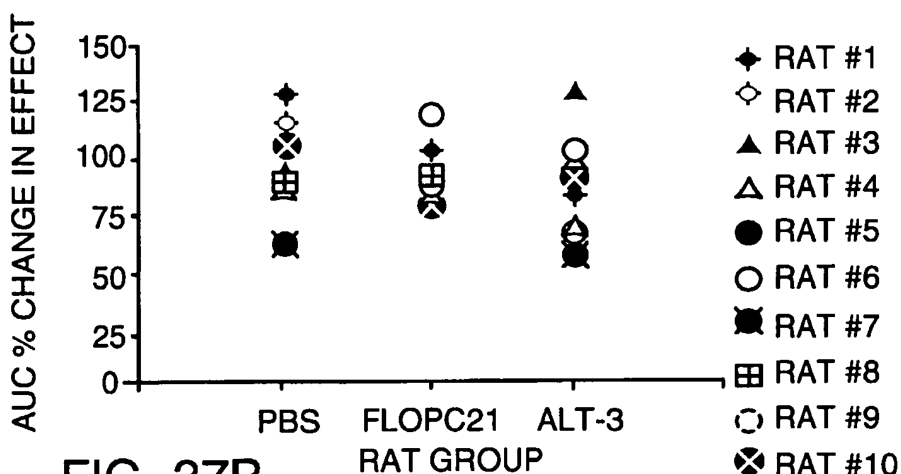


FIG. 37B

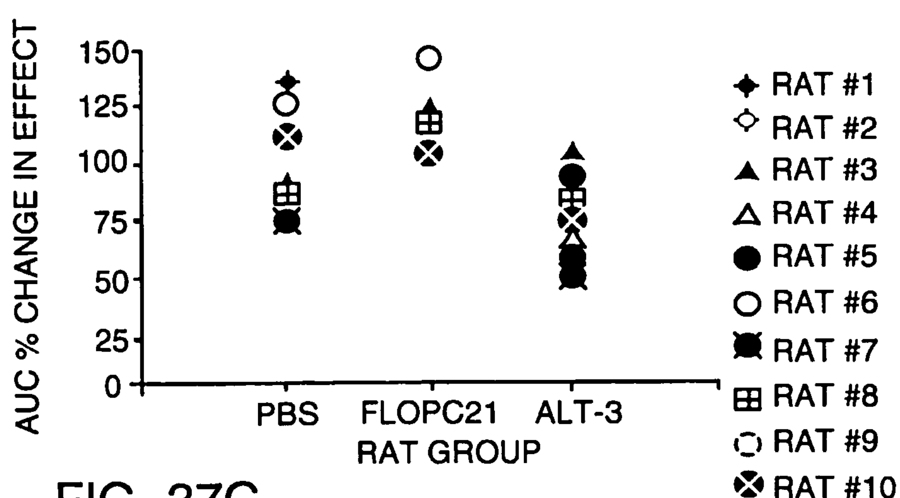


FIG. 37C

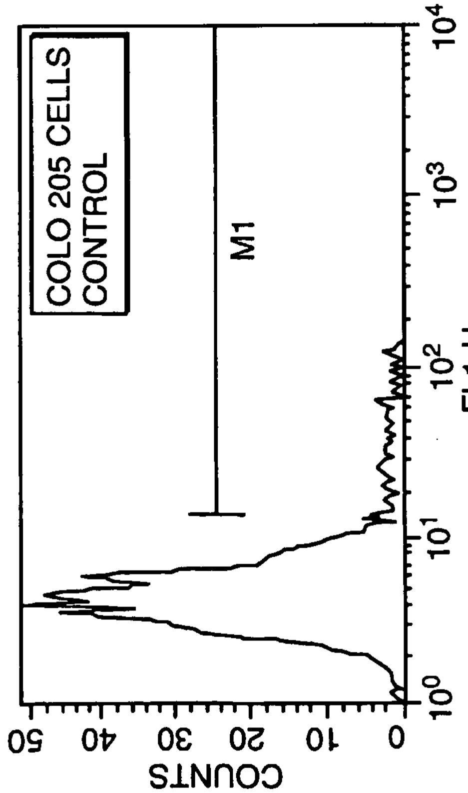


FIG. 38A

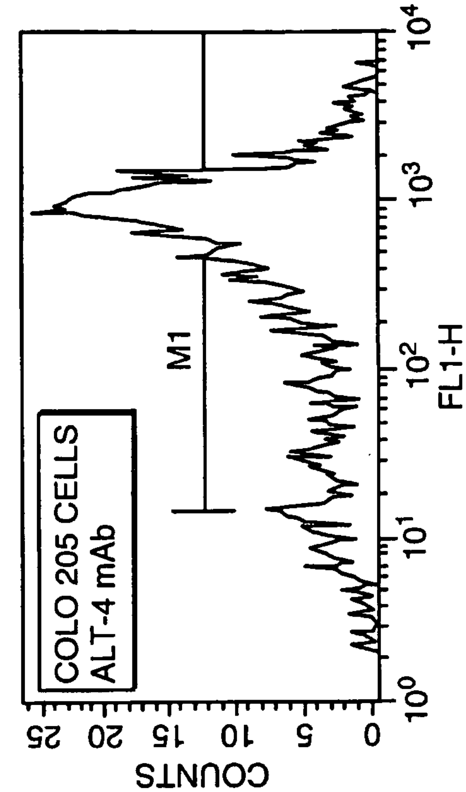


FIG. 38B

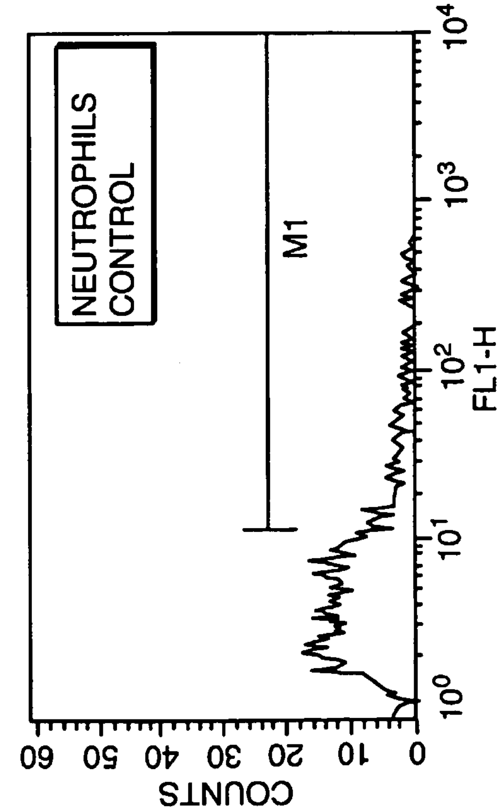


FIG. 38C

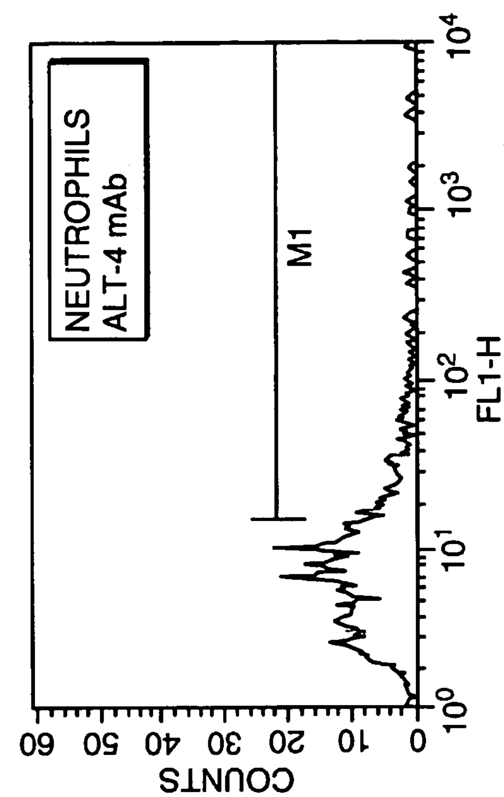


FIG. 38D

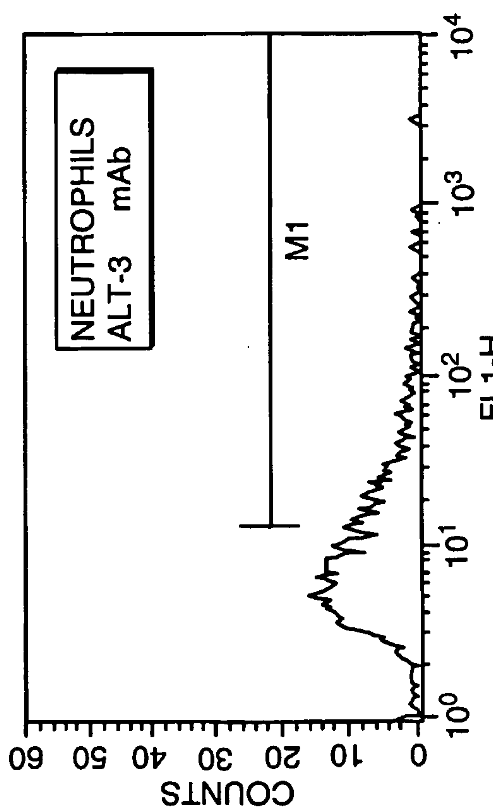


FIG. 38E

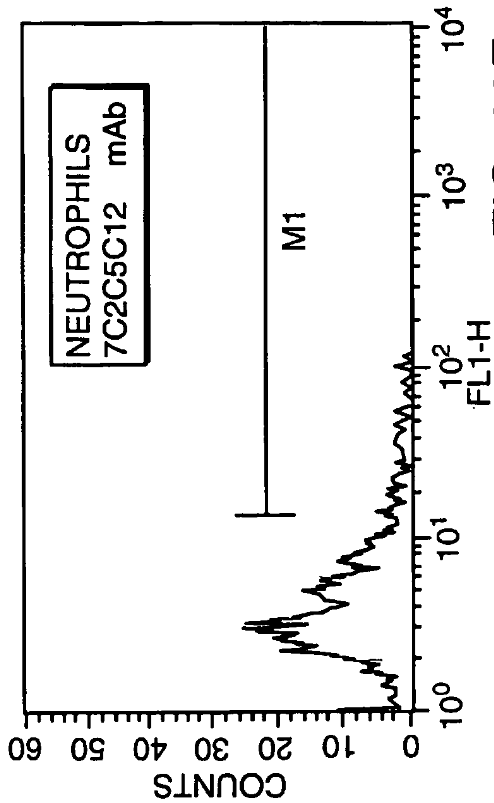


FIG. 38F

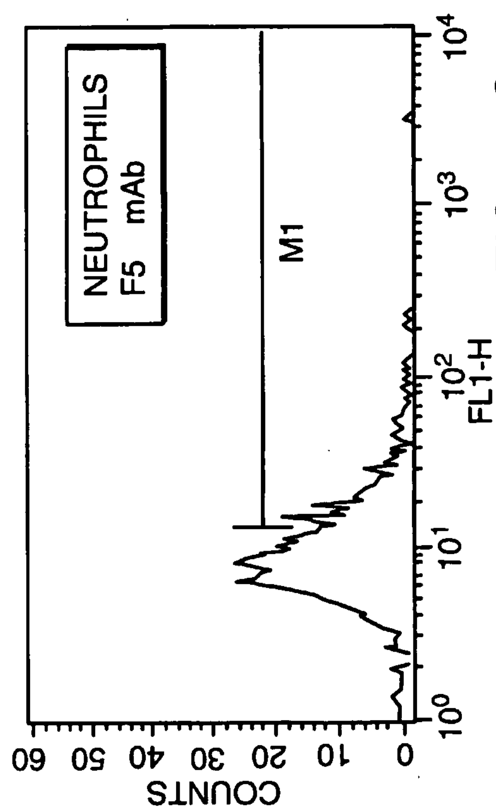


FIG. 38G

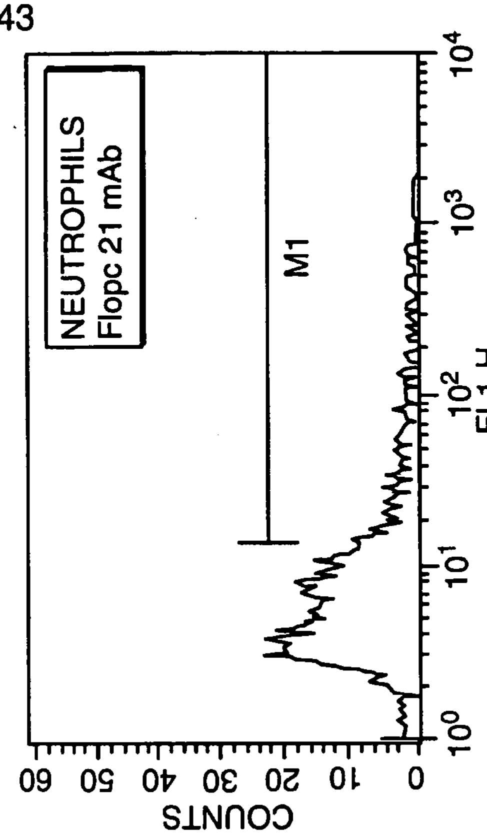


FIG. 38H

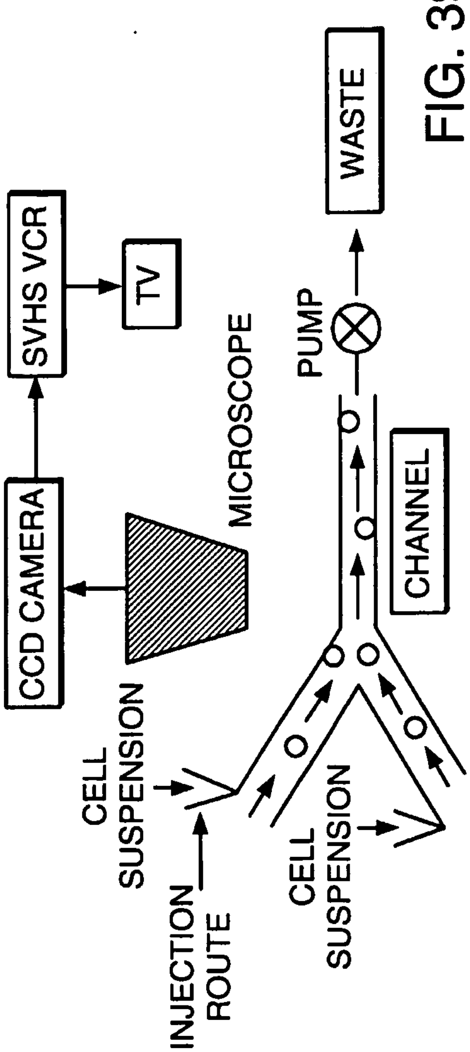


FIG. 39A

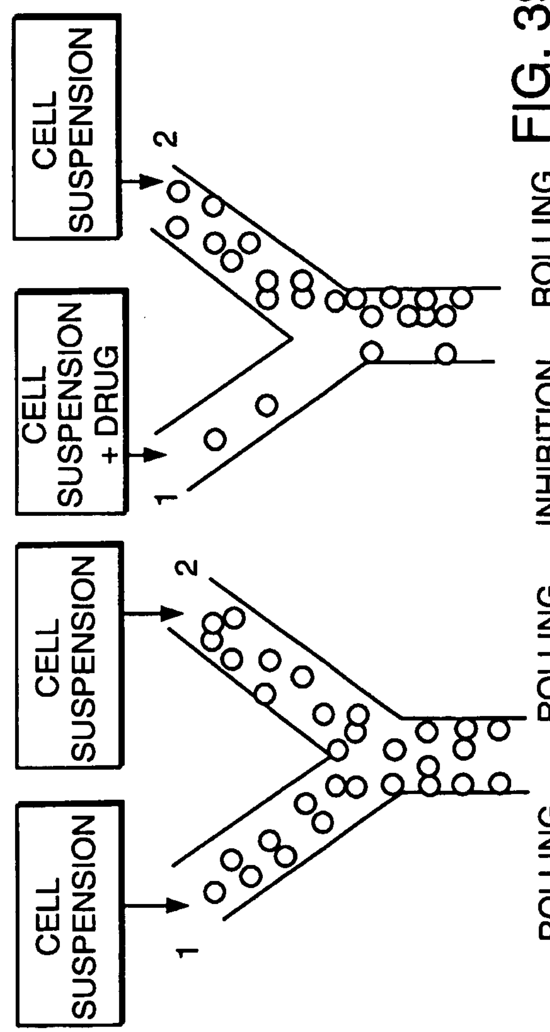


FIG. 39B

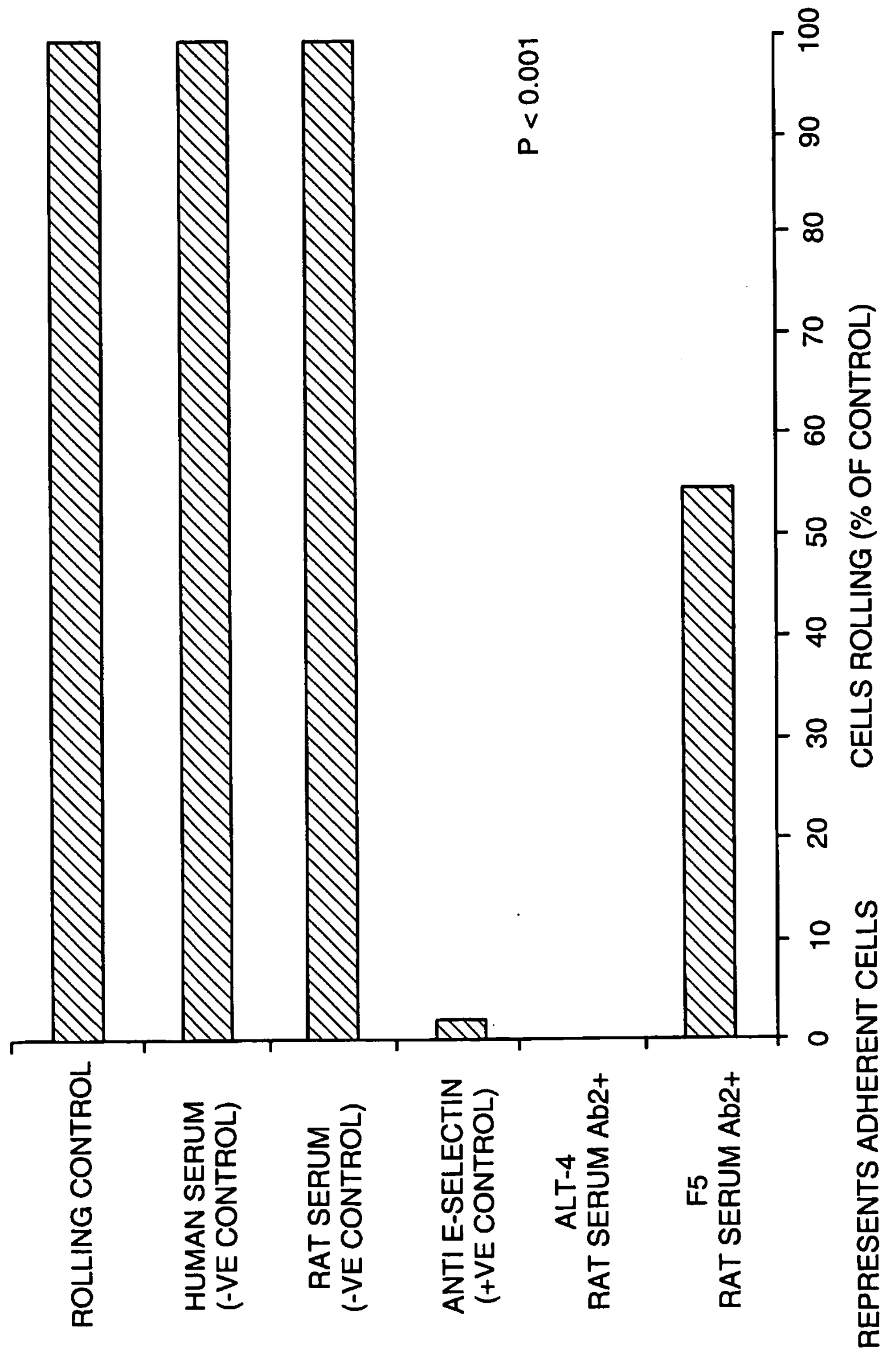

P < 0.001
ROLLING CONTROL
HUMAN SERUM (-VE CONTROL)
RAT SERUM (-VE CONTROL)
ANTI E-SELECTIN (+VE CONTROL)
ALT-4 RAT SERUM Ab2+
F5 RAT SERUM Ab2+
REPRESENTS ADHERENT CELLS
0
10
20
30
40
50
60
70
80
90
100
CELLS ROLLING (% OF CONTROL)

FIG. 40

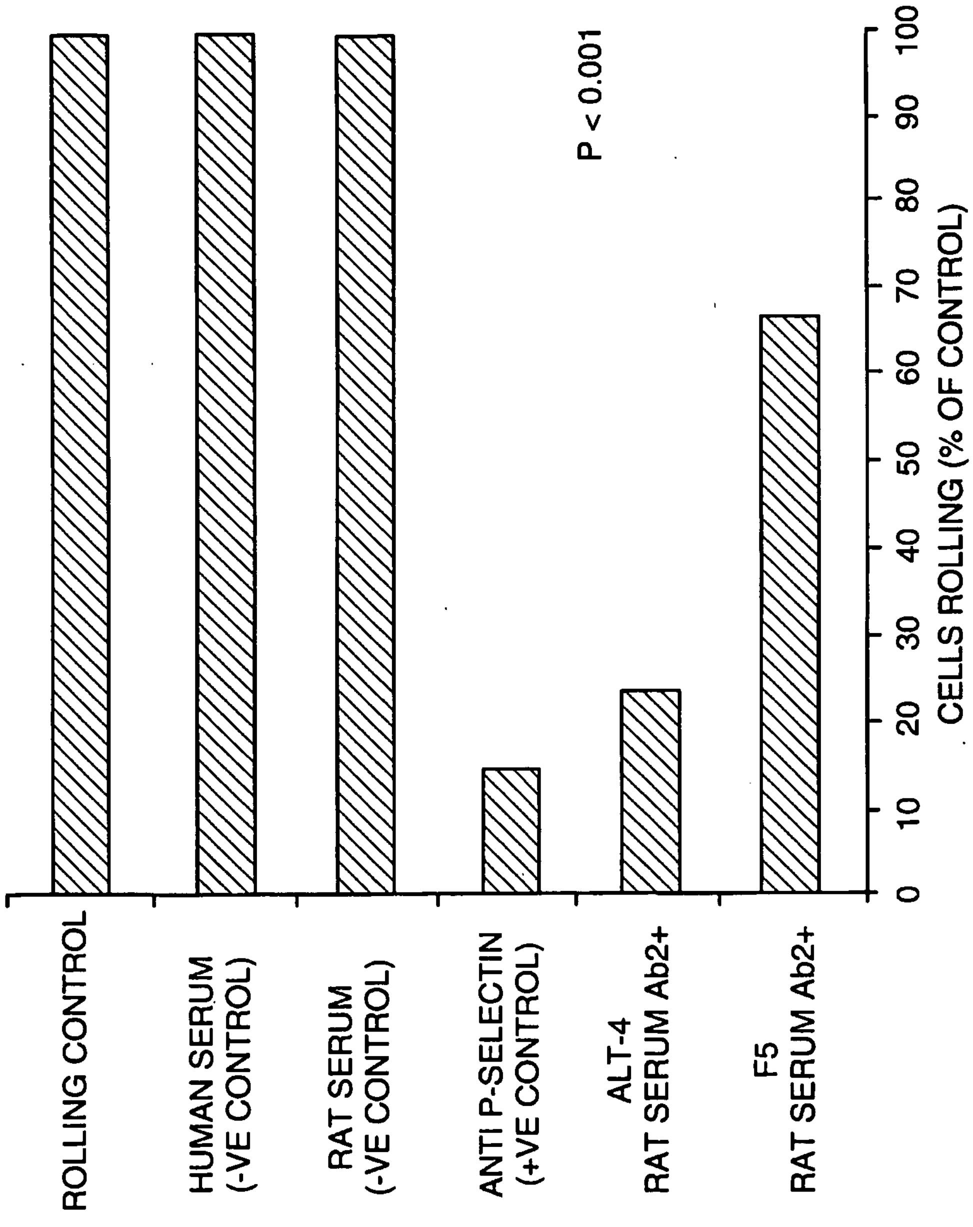

ROLLING CONTROL
HUMAN SERUM (-VE CONTROL)
RAT SERUM (-VE CONTROL)
ANTI P-SELECTIN (+VE CONTROL)
ALT-4 RAT SERUM Ab2+
F5 RAT SERUM Ab2+
P < 0.001
0
10
20
30
40
50
60
70
80
90
100
CELLS ROLLING (% OF CONTROL)

FIG. 41